FORM PTO-1390 (Modified) (REV 10-95) U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE

TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371

ATTORNEY'S DOCKET NUMBER: **MUR-7520**

U.S. APPLICATION NO. (IF KNOWN, SEE 37 CFR): **09/155708**

INTERNATIONAL APPLICATION NO.	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED
PCT/GB97/00929	**2 April 1997**	**2 April 1996**

TITLE OF INVENTION

GENETIC SUPPRESSION AND REPLACEMENT

APPLICANT(S) FOR DO/EO/US

Gwenyth Jane Farrar, Peter Humphries, and Paul Francis Kenna

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. ☐ This is an express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(1).
4. ☒ A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.
5. ☒ A copy of the International Application as filed (35 U.S.C. 371 (c) (2))
   a. ☒ is transmitted herewith (required only if not transmitted by the International Bureau).
   b. ☐ has been transmitted by the International Bureau.
   c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US).
6. ☐ A translation of the International Application into English (35 U.S.C. 371(c)(2)).
7. ☒ A copy of the International Search Report (PCT/ISA/210).
8. ☒ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371 (c)(3))
   a. ☐ are transmitted herewith (required only if not transmitted by the International Bureau).
   b. ☐ have been transmitted by the International Bureau.
   c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
   d. ☒ have not been made and will not be made.
9. ☐ A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
10. ☐ An oath or declaration of the inventor(s) (35 U.S.C. 371 (c)(4)).
11. ☒ A copy of the International Preliminary Examination Report (PCT/IPEA/409).
12. ☐ A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371 (c)(5)).

Items 13 to 18 below concern document(s) or information included:

13. ☐ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
14. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
15. ☒ A **FIRST** preliminary amendment.
    A **SECOND** or **SUBSEQUENT** preliminary amendment.
16. ☐ A substitute specification.
17. ☐ A change of power of attorney and/or address letter.
18. ☒ Certificate of Mailing by Express Mail
19. ☒ Other items or information:

Combined Declaration and Power of Attorney (unexecuted)
Executed Verified Statement Claiming Small Entity Status - Nonprofit Organization
Verified Statement Claiming Small Entity Status - Independent Inventor (unexecuted) (for informational purposes)
Verified Statement Claiming Small Entity Status - Nonprofit Organization (unexecuted) (for informational purposes)

U.S. APPLICATION NO. (IF KNOWN, SEE 37 CFR	INTERNATIONAL APPLICATION NO. PCT/GB97/00929	ATTORNEY'S DOCKET NUMBER MUR-7520

20. The following fees are submitted:.

BASIC NATIONAL FEE (37 CFR 1.492 (a) (1) - (5)) :

		CALCULATIONS PTO USE ONLY
☒	Search Report has been prepared by the EPO or JPO	**$930.00**
☐	International preliminary examination fee paid to USPTO (37 CFR 1.482)	**$720.00**
☐	No international preliminary examination fee paid to USPTO (37 CFR 1.482) but international search fee paid to USPTO (37 CFR 1.445(a)(2))	**$790.00**
☐	Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2) paid to USPTO	**$1,070.00**
☐	International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(2)-(4)	**$98.00**

ENTER APPROPRIATE BASIC FEE AMOUNT =		**$930.00**
Surcharge of **$130.00** for furnishing the oath or declaration later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492 (e)).		**$0.00**

CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE	
Total claims	11 - 20 =	0	x $22.00	**$0.00**
Independent claims	5 - 3 =	2	x $82.00	**$164.00**
Multiple Dependent Claims **(check if applicable)**.			☐	**$0.00**
TOTAL OF ABOVE CALCULATIONS			=	**$1,094.00**
Reduction of 1/2 for filing by small entity, if applicable. Verified Small Entity Statement must be filed (Note 37 CFR 1.9, 1.27, 1.28) **(check if applicable)**.			☒	**$547.00**
SUBTOTAL			=	**$547.00**
Processing fee of **$130.00** for furnishing the English translation later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492 (f)).			+	**$0.00**
TOTAL NATIONAL FEE			=	**$547.00**
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31) **(check if applicable).**			☐	**$0.00**
TOTAL FEES ENCLOSED			=	**$547.00**
			Amount to be: refunded	$
			charged	$

☒ A check in the amount of **$547.00** to cover the above fees is enclosed.

☐ Please charge my Deposit Account No. in the amount of to cover the above fees.
A duplicate copy of this sheet is enclosed.

☒ The Commissioner is hereby authorized to charge any fees which may be required, or credit any overpayment to Deposit Account No. **18-0350** A duplicate copy of this sheet is enclosed.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:

Allan Ratner
Ratner & Prestia
Suite 301
One Westlakes, Berwyn
P.O. Box 980
Valley Forge, PA 19482-0980
(610) 407-0700

SIGNATURE

Allan Ratner
NAME

19,717
REGISTRATION NUMBER

October 2, 1998
DATE

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant:	Gwenyth Jane Farrar, Peter Humphries, and Paul Francis Kenna	: Interntl Serial No.: PCT/GB97/00929
Serial No.:	(to be assigned)	:
Filed:	(herewith)	: Interntl Filing Date:
FOR:	GENETIC SUPPRESSION AND REPLACEMENT	: 2 April 1997

PRELIMINARY AMENDMENT

Assistant Commissioner for Patents
Washington, D.C. 20231

S I R :

Preliminary to examination in the United States Patent and Trademark Office, please make the following amendments in the above-identified application in order to place it in condition for examination.

Amend the specification by inserting before the first line the sentence:

This application is the U.S. national phase application of PCT International Application No. PCT/GB97/00929 filed 2 April 1997.

IN THE CLAIMS:

Please amend the claims as follows:

5. (Amended) The use of a strategy as claimed in [any of the preceding] Claim[s] 1 in the preparation of a medicament for the treatment of an autosomal dominant disease caused by an endogenous target gene wherein the disease is caused by different mutations in the same gene in different patients.

7. (Amended) A use as claimed in Claim[s 5 or] 6 wherein the disease is a polygenic disorder.

8. (Amended) A use as claimed in Claim 6 [or 7] wherein suppressor(s) or replacement gene(s) are administered alone or in vector(s) chosen from DNA plasmid vectors, RNA or DNA viral vectors.

11. (Amended) A use as claimed [as] in Claim[s] 1 [to 10] wherein the replacement gene is altered from the wild type gene and provides a beneficial effect when compared to the wild type gene.

Respectfully submitted,

Allan Rather, Reg. No. 19,717
Attorney for Applicant

AR/lk

Dated: October 2, 1998

P.O. Box 980
Valley Forge, PA 19482
(610) 407-0700

The Assistant Commissioner for Patents is hereby authorized to charge payment to Deposit Account No. **18-0350** of any fees associated with this communication.

EXPRESS MAIL Mailing Label Number: **EM009003534US**
Date of Deposit: **October 2, 1998**

I hereby certify that this paper and fee are being deposited, under 37 C.F.R. § 1.10 and with sufficient postage, using the "Express Mail Post Office to Addressee" service of the United States Postal Service on the date indicated above and that the deposit is addressed to the Assistant Commissioner for Patents, U.S. Patent & Trademark Office, Washington, D.C. 20231, Attn: BOX PCT/EO/US.

Kathleen Libby
Kathleen Libby

Applicant or Patentee: Gwyneth Jane Farrar, Peter Humphries, and Paul Francis Kenna
Serial or Patent No: U.S. National Phase of PCT/GB97/00929
Filed or Issued: International Filing Date 2 April 1997.
For: GENETIC SUPPRESSION AND REPLACEMENT.

Attorney's Docket No.: MUR-712P. #3

VERIFIED STATEMENT (DECLARATION) CLAIMING SMALL ENTITY STATUS (37 CFR 1.9(f) and 1.27(d)) - NONPROFIT ORGANIZATION

I hereby declare that I am an official empowered to act on behalf of the nonprofit organization identified below:

NAME OF ORGANIZATION: Provost, Fellows and Scholars of the College of the Holy and Undivided Trinity of Queen Elizabeth near Dublin

ADDRESS OF ORGANIZATION: Trinity College, Dublin 2, IRELAND

TYPE OF ORGANIZATION ____

- ☐ University or other institution of higher education
- ☐ Tax exempt under Internal Revenue Service Code (26 USC 501(a) and 501(c)(3))
- ☐ Nonprofit scientific or educational under statute of state of the United States of America
  (Name of state)
  (Citation of statute .)
- ☐ Would qualify as tax exempt under Internal Revenue Service Code (26 USC 501(a) and 501(c)(3)) if located in the United States of America
- ☐ Would qualify as nonprofit scientific or educational under statute of state of the United States of America if located in the United States of America
  (Name of state)
  (Citation of statute .)

I hereby declare that the nonprofit organization identified qualifies as a nonprofit organization as defined in 37 CFR 1.9(e) for purposes of paying reduced fees under section 41(a) and (b) of Title 35, United States Code with regard to the invention entitled GENETIC SUPPRESSION AND REPLACEMENT by inventor(s) Gwyneth Jane Farrar, Peter Humphries, and Paul Francis Kenna described in

- ☐ the specification filed herewith.
- ☒ application serial no. PCT/GB97/00929, filed 2 April 1997.
- ☐ patent no. ____, issued ____

I hereby declare that rights under contract or law have been conveyed to and remain with the nonprofit organization with regard to the above identified invention.

If the rights held by the nonprofit organization are not exclusive, each individual, concern or organization having rights to the invention is listed below* and no rights to the invention are held by any person, other than the inventor, who could not qualify as a small business concern under 37 CFR 1.9(d) or by any concern which would not qualify as a small business concern under 37 CFR 1.9(d) or a nonprofit organization under 37 CFR 1.9(e).

*NOTE: Separate verified statements are required from each named person, concern or organization having rights to the invention averring to their status as small entities. (37 CFR 1.27)

NAME ____
ADDRESS ____
☐ INDIVIDUAL ☐ SMALL BUSINESS CONCERN ☐ NONPROFIT ORGANIZATION

NAME ____
ADDRESS ____
☐ INDIVIDUAL ☐ SMALL BUSINESS CONCERN ☐ NONPROFIT ORGANIZATION

NAME ____
ADDRESS ____
☐ INDIVIDUAL ☐ SMALL BUSINESS CONCERN ☐ NONPROFIT ORGANIZATION

I acknowledge the duty to file, in this application or patent, notification of any change in status resulting in loss of entitlement to small entity status prior to paying, or at the time of paying, the earliest of the issue fee or any maintenance fee due after the date on which status as a small business entity is no longer appropriate. (37 CFR 1.28(b)).

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this verified statement is directed.

NAME OF PERSON SIGNING: Eoin P. O'Neill EOIN P. O'NEILL
TITLE OF PERSON OTHER THAN OWNER: DIRECTOR OF INNOVATION SERVICES
ADDRESS OF PERSON SIGNING: TRINITY COLLEGE DUBLIN 2 IRELAND
SIGNATURE: Eoin P. O'Neill DATE: 2ND OCT '98

Applicant or Patentee: Gwenyth Jane Farrar, Peter Humphries, and Paul Francis Kenna
Serial or Patent No.: U.S. National Phase of PCT/GB97/00929
Filed or Issued: International Filing Date: 2 April 1997.
For: GENETIC SUPPRESSION AND REPLACEMENT .

Attorney's Docket No.: MUR-7520 .

VERIFIED STATEMENT (DECLARATION) CLAIMING SMALL ENTITY STATUS (37 CFR 1.9(f) and 1.27(b) - INDEPENDENT INVENTOR

As a below named inventor, I hereby declare that I qualify as an independent inventor as defined in 37 CFR 1.9(c) for purposes of paying reduced fees under section 41(a) and (b) of Title 35, United States Code, to the Patent and Trademark Office with regard to the invention entitled GENETIC SUPPRESSION AND REPLACEMENT described in

☐ the specification filed herewith.

☒ application serial no. PCT/GB97/00929, filed 2 April 1997

☐ patent no. ___, issued ___

I have not assigned, granted, conveyed or licensed and am under no obligation under contract or law to assign, grant, convey or license, any rights in the invention to any person who could not be classified as an independent inventor under 37 CFR 1.9(c) if that person had made the invention, or to any concern which would not qualify as a small business concern under 37 CFR 1.9(d) or a nonprofit organization under 37 CFR 1.9(e).

Each person, concern or organization to which I have assigned, granted, conveyed, or licensed or am under an obligation under contract or law to assign, grant, convey, or license any rights in the invention is listed below:

☐ no such person, concern, or organization

☐ persons, concerns, or organizations listed below*

*NOTE: Separate verified statements are required from each named person, concern or organization having rights to the invention averring to their status as small entities. (37 CFR 1.27)

FULL NAME _____

ADDRESS _____

☐ INDIVIDUAL ☐ SMALL BUSINESS CONCERN ☐ NONPROFIT ORGANIZATION

FULL NAME _____

ADDRESS _____

☐ INDIVIDUAL ☐ SMALL BUSINESS CONCERN ☐ NONROFIT ORGANIZATION

FULL NAME _____

ADDRESS _____

☐ INDIVIDUAL ☐ SMALL BUSINESS CONCERN ☐ NONPROFIT ORGANIZATION

I acknowledge the duty to file, in this application or patent, notification of any change in status resulting in loss of entitlement to small entity status prior to paying, or at the time of paying, the earliest of the issue fee or any maintenance fee due after the date on which status as a small entity is no longer appropriate. (37 CFR 1.28(b)).

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this verified statement is directed.

Gwenyth Jane Farrar	Peter Humphries	Paul Francis Kenna .
NAME OF INVENTOR	NAME OF INVENTOR	NAME OF INVENTOR
_____	_____	_____
Signature of Inventor	Signature of Inventor	Signature of Inventor
_____	_____	_____
Date	Date	Date

Applicant or Patentee: Gwenyth Jane Farrar, Peter Humphries, and Paul Francis Kenna
Serial or Patent No.: U.S. National Phase of PCT/GB97/00929
Filed or Issued: International Filing Date: 2 April 1997.
For: GENETIC SUPPRESSION AND REPLACEMENT .

Attorney's Docket No.: MUR-7520 .

VERIFIED STATEMENT (DECLARATION) CLAIMING SMALL ENTITY STATUS (37 CFR 1.9(f) and 1.27(d) - NONPROFIT ORGANIZATION

I hereby declare that I am an official empowered to act on behalf of the nonprofit organization identified below:

NAME OF ORGANIZATION Provost, Fellows and Scholars of the College of the Holy and Undivided Trinity of Queen Elizabeth near Dublin

ADDRESS OF ORGANIZATION Trinity College, Dublin 2, IRELAND

TYPE OF ORGANIZATION

- ☐ University or other institution of higher education
- ☐ Tax exempt under Internal Revenue Service Code (26 USC 501(a) and 501(c)(3))
- ☐ Nonprofit scientific or educational under statute of state of the United States of America
  (Name of state __)
  (Citation of statute __)
- ☐ Would qualify as tax exempt under Internal Revenue Service Code (26 USC 501(a) and 501(c) (3)) if located in the United States of America
- ☐ Would qualify as nonprofit scientific or educational under statute of state of the United States of America if located in the United States of America
  (Name of state __)
  (Citation of statute __)

I hereby declare that the nonprofit organization identified qualifies as a nonprofit organization as defined in 37 CFR 1.9(e) for purposes of paying reduced fees under section 41(a) and (b) of Title 35, United States Code with regard to the invention entitled GENETIC SUPPRESSION AND REPLACEMENT by inventor(s) Gwenyth Jane Farrar, Peter Humphries, and Paul Francis Kenna described in

- ☐ the specification filed herewith.
- ☒ application serial no. PCT/GB97/00929 , filed 2 April 1997.
- ☐ patent no. __ , issued __.

I hereby declare that rights under contract or law have been conveyed to and remain with the nonprofit organization with regard to the above identified invention.

If the rights held by the nonprofit organization are not exclusive, each individual, concern or organization having rights to the invention is listed below* and no rights to the invention are held by any person, other than the inventor, who could not qualify as a small business concern under 37 CFR 1.9(d) or by any concern which would not qualify as a small business concern under 37 CFR 1.9(d) or a nonprofit organization under 37 CFR 1.9(e).

*NOTE: Separate verified statements are required from each named person, concern or organization having rights to the invention averring to their status as small entities. (37 CFR 1.27)

NAME _____

ADDRESS _____

☐ INDIVIDUAL ☐ SMALL BUSINESS CONCERN ☐ NONPROFIT ORGANIZATION

NAME _____

ADDRESS _____

☐ INDIVIDUAL ☐ SMALL BUSINESS CONCERN ☐ NONPROFIT ORGANIZATION

NAME _____

ADDRESS _____

☐ INDIVIDUAL ☐ SMALL BUSINESS CONCERN ☐ NONPROFIT ORGANIZATION

I acknowledge the duty to file, in this application or patent, notification of any change in status resulting in loss of entitlement to small entity status prior to paying, or at the time of paying, the earliest of the issue fee or any maintenance fee due after the date on which status as a small business entity is no longer appropriate. (37 CFR 1.28(b)).

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this verified statement is directed.

NAME OF PERSON SIGNING _____

TITLE OF PERSON OTHER THAN OWNER _____

ADDRESS OF PERSON SIGNING _____

SIGNATURE _____ DATE _____

09/155708
10 Rec'd PCT/PTO 02 OCT 1998
PCT/GB97/00929

"Genetic Suppression and Replacement"

The present invention relates to a strategy for suppressing a gene. In particular the invention relates to suppression of mutated genes which give rise to a dominant or deleterious effect, either monogenically or polygenically. The invention relates to a strategy for suppressing a gene or disease allele using methods which do not target the disease allele specifically but instead can be targeted towards a broad range of sequences in a particular gene. A particular embodiment of the invention is the use of suppression strategies to target either the disease or normal alleles alone or to target both disease and normal alleles. A further embodiment of the invention is the use of the wobble hypothesis to enable continued expression of a replacement normal or beneficial gene (a gene modified from the wild type such that it provides an additional beneficial effect(s)). The replacement gene will have nucleotide changes from the endogenous wild type gene but will code for identical amino acids as the wild type gene. The strategy circumvents the need for a specific therapy for every mutation within a given gene. In addition the invention allows greater flexibility in choice of

target sequence for suppression of a disease allele.

The invention also relates to a medicament or medicaments for use in suppressing a deleterious allele which is present in a genome of one or more individuals or animals.

Generally the present invention will be useful where the gene, which is naturally present in the genome of a patient, contributes to a disease state. Generally, one allele of the gene in question will be mutated, that is, will possess alterations in its nucleotide sequence that affects the function or level of the gene product. For example, the alteration may result in an altered protein product from the wild type gene or altered control of transcription and processing. Inheritance or somatic acquisition of such a mutation can give rise to a disease phenotype or can predispose an individual to a disease phenotype. However the gene of interest could also be of wild type phenotype, but contribute to a disease state in another way such that the suppression of the gene would alleviate or improve the disease state or improve the effectiveness of an administered therapeutic compound.

Generally, suppression effectors such as nucleic acids - antisense or sense, ribozymes, peptide nucleic acids (PNAs), triple helix forming oligonucleotides, peptides and /or antibodies directed to sequences in a gene, in transcripts or in protein, can be employed in the invention to achieve gene suppression.

BACKGROUND

Studies of degenerative hereditary ocular conditions, including Retinitis Pigmentosa (RP) and various macular

dystrophies have resulted in a substantial elucidation of the molecular basis of these debilitating human retinal degenerations. Applying the approach of genetic linkage, x-linked RP (xlRP) genes have been localised to the short arm of the X chromosome (Ott et al. 1990) - subsequently the gene involved in one form of xlRP has been identified. Various genes involved in autosomal dominant forms of RP (adRP) have been localised. The first of these mapped on 3q close to the gene encoding the rod photoreceptor protein rhodopsin (McWilliam et al. 1989; Dryja et al. 1990). Similarly, an adRP gene was placed on 6p close to the gene encoding the photoreceptor protein peripherin (Farrar et al. 1991a,b; Kajiwara et al. 1991). Other adRP genes have been mapped to discrete chromosomal locations however the disease genes as yet remain uncharacterised. As in xlRP and adRP, various genes involved in autosomal recessive RP (arRP) have been localised and in some cases molecular defects characterised (Humphries et al. 1992; Farrar et al. 1993; Van Soest et al. 1994). Similarly a number of genes involved in macular dystrophies have been mapped (Mansergh et al. 1995). Genetic linkage, together with techniques for mutational screening of candidate genes, enabled identification of causative dominant mutations in the genes encoding rhodopsin and peripherin. Globally about 100 rhodopsin mutations have been found in patients with RP or congenital stationary night blindness. Similarly approximately 40 mutations have been characterised in the peripherin gene in patients with RP or macular dystrophies. Knowledge of the molecular aetiology of these retinopathies has stimulated the generation of animal models and the exploration of methods of therapeutic intervention (Farrar et al. 1995; Humphries et al. 1997).

Similar to RP, osteogenesis imperfecta (OI) is an autosomal dominantly inherited human disorder whose molecular pathogenesis is extremely genetically heterogeneous. OI is often referred to as 'brittle bone disease' although additional symptoms including hearing loss, growth deficiency, bruising, loose joints, blue sclerae and dentinogenesis imperfeca are frequently observed (McKusick, 1972). Mutations in the genes encoding the two type I collagen chains (collagen 1A1 and 1A2) comprising the type I collagen heterodimer have been implicated in OI. Indeed hundreds of dominantly acting mutations have been identified in OI patients in these two genes, many of which are single point mutations, although a number of insertion and deletion mutations have been found (Willing et al. 1993; Zhuang et al. 1996). Similarly mutations in these genes have also been implicated in Ehlers-Danlos and Marfan syndromes (Dalgleish et al. 1986; Phillips et al. 1990; D'Alessio et al. 1991; Vasan NS et al. 1991).

Generally, gene therapies utilising viral and non-viral delivery systems have been used to treat inherited disorders, cancers and infectious diseases. However, many studies have focused on recessively inherited disorders, the rationale being that introduction and expression of the wild type gene may be sufficient to prevent/ameliorate the disease phenotype. In contrast gene therapy for dominant disorders will require suppression of the dominant disease allele. Notably many of the characterised mutations causing inherited diseases such as RP or OI are inherited in an autosomal dominant fashion. Indeed there are over 1,000 autosomal dominantly inherited disorders in man. In addition there are many polygenic disorders due to co-inheritance of a number of genetic components

which together give rise to the disease state. Effective gene therapies for dominant or polygenic diseases may be targeted to the primary defect and in this case may require suppression of the disease allele while in many cases still maintaining the function of the normal allele. This will be particularly relevant where disease pathology is due to a gain of function mutation rather than due to reduced levels of wild type protein. Alternatively suppression therapies may be targeted to secondary effects associated with the disease pathology: one example is programmed cell death (apoptosis) which has been observed in many inherited disorders.

Strategies to differentiate between normal and disease alleles and to selectively switch off the disease allele using suppression effectors inter alia antisense DNA/RNA, PNAs, ribozymes or triple helix DNA, targeted towards the disease mutation may be difficult in many cases - frequently disease and normal alleles differ by only a single nucleotide. A further difficulty inhibiting development of gene therapies is the heterogeneous nature of some dominant disorders - many different mutations in the same gene give rise to a similar disease phenotype. Development of specific gene therapies for each of these may be prohibitive in terms of cost. To circumvent difficulties associated with specifically targeting the disease mutation and with the genetic heterogeneity present in inherited disorders, a novel strategy for gene suppression and gene replacement exploiting the degeneracy of the genetic code, thereby allowing flexibility in choice of target sequence for suppression and providing a means of gene suppression which is independent of the disease mutation, is described in the invention.

PRIOR ART

Suppression effectors have been used previously to achieve specific suppression of gene expression. Antisense DNA and RNA has been used to inhibit gene expression in many instances. Modifications, such as phosphorothioates, have been made to oligonucleotides to increase resistance to nuclease degradation, binding affinity and uptake (Cazenave et al. 1989; Sun et al. 1989; McKay et al. 1996; Wei et al. 1996). In some instances, using antisense and ribozyme suppression strategies has led to reversal of a tumour phenotype by reducing expression of a gene product or by cleaving a mutant transcript at the site of the mutation (Carter and Lemoine 1993; Lange et al. 1993; Valera et al. 1994; Dosaka-Akita et al. 1995; Feng et al. 1995; Quattrone et al. 1995; Ohta et al. 1996). For example, neoplastic reversion was obtained using a ribozyme targeted to a H-ras mutation in bladder carcinoma cells (Feng et al. 1995). Ribozymes have also been proposed as a means of both inhibiting gene expression of a mutant gene and of correcting the mutant by targeted *trans*-splicing (Sullenger and Cech 1994; Jones et al. 1996). Ribozymes can be designed to elicit autocatalytic cleavage of RNA targets, however, the inhibitory effect of some ribozymes may be due in part to an antisense effect due to the antisense sequences flanking the catalytic core which specify the target site (Ellis and Rodgers 1993; Jankowsky and Schwenzer 1996). Ribozyme activity may be augmented by the use of, for example, non-specific nucleic acid binding proteins or facilitator oligonucleotides (Herschlag et al. 1994; Jankowsky and Schwenzer 1996). Multitarget ribozymes (connected or shotgun) have been suggested as a means of improving efficiency of ribozymes for gene suppression (Ohkawa et al. 1993). Triple helix

approaches have also been investigated for sequence specific gene suppression - triplex forming oligonucleotides have been found in some cases to bind in a sequence specific manner (Postel et al. 1991; Duval-Valentin et al. 1992; Hardenbol and Van Dyke 1996; Porumb et al. 1996). Similarly peptide nucleic acids have been shown to inhibit gene expression (Hanvey et al. 1992; Knudson and Nielsen 1996; Taylor et al. 1997). Minor groove binding polyamides can bind in a sequence specific manner to DNA targets and hence may represent useful small molecules for future suppression at the DNA level (Trauger et al. 1996). In addition, suppression has been obtained by interference at the protein level using dominant negative mutant peptides and antibodies (Herskowitz 1987; Rimsky et al. 1989; Wright et al. 1989). In some cases suppression strategies have lead to a reduction in RNA levels without a concomitant reduction in proteins, whereas in others, reductions in RNA have been mirrored by reductions in protein.

There is now an armament with which to obtain gene suppression. This, in conjunction with a better understanding of the molecular etiology of disease, results in an ever increasing number of disease targets for therapies based on suppression. In many cases, complete suppression of gene expression has been difficult to achieve. Possibly a combined approach using a number of suppression effectors may aid in this. For some disorders it may be necessary to block expression of a disease allele completely to prevent disease symptoms whereas for others low levels of mutant protein may be tolerated. In parallel with an increased knowledge of the molecular defects causing disease has been the realisation that many disorders are genetically heterogeneous. Examples in which

multiple genes and/or multiple mutations within a gene can give rise to a similar disease phenotype include osteogenesis imperfecta, familial hypercholesteraemia, retinitis pigmentosa, and many others. The utility of the degeneracy of the genetic code (wobble hypothesis) to enable suppression of one or both alleles of a gene and the introduction of a replacement gene such that it escapes suppression has been exploited in the invention.

The invention addresses shortcomings of the prior art by providing a novel approach to the design of suppression effectors directed to target alleles of a gene carrying a deleterious mutation. Suppression of every mutation giving rise to a disease phenotype may be costly and problematic. Disease mutations are often single nucleotide changes. As a result differentiating between the disease and normal alleles may be difficult. Some suppression effectors require specific sequence targets, for example, hammerhead ribozymes cleave at NUX sites and hence may not be able to target many mutations. Notably, the wide spectrum of mutations observed in many diseases adds additional complexity to the development of therapeutic strategies for such disorders - some mutations may occur only once in a single patient. A further problem associated with suppression is the high level of homology present in coding sequences between members of some gene families. This can limit the range of target sites for suppression which will enable specific suppression of a single member of such a gene family.

The present invention circumvents shortcomings in the prior art by utilising the degeneracy of the genetic code. In the invention suppression effectors are designed specifically to sequences in coding regions of

genes or in gene products. Typically one allele of the gene contains a mutation with a deleterious effect. Suppression targeted to coding sequences provides greater flexibility in choice of target sequence for suppression in contrast to suppression directed towards single disease mutations. Additionally the invention provides for the introduction of a replacement gene with modified sequences such that the replacement gene is protected from suppression. The replacement gene is modified at third base wobble positions and hence provides the wild type gene product. Notably, the invention has the advantage that the same suppression strategy could be used to suppress, in principle, many mutations in a gene. This is particularly relevant when large numbers of mutations within a single gene cause disease pathology. The replacement gene provides (when necessary) expression of the normal protein product when required to ameliorate pathology associated with reduced levels of wild type protein. The same replacement gene could in principle be used in conjunction with the suppression of many different disease mutations within a given gene. Target sequences may be found in any part of the coding sequence. Suppression in coding sequence holds the advantage that such sequences are present in both precursor and mature RNAs, thereby enabling suppressors to target all forms of RNA.

In summary the invention involves gene suppression of disease and normal alleles targeting coding sequences in a gene and when necessary gene replacement such that the replacement gene cannot be suppressed. Replacement genes are modified at third base positions (wobble positions) so that they code for the correct amino acids but are protected completely or partially from suppression. The same suppression and replacement

steps can be used for many disease mutations in a given gene. Suppression and replacement can be undertaken in conjunction with each other or separately.

DESCRIPTION OF INVENTION

According to the present invention there is provided a strategy for suppressing expression of an endogenous gene with a deleterious mutation, wherein said strategy comprises providing suppression effectors such as antisense nucleic acids able to bind to sequences of a gene to be suppressed, to prevent the functional expression thereof.

Generally the term suppression effectors means nucleic acids, peptide nucleic acids (PNAs), peptides, antibodies or modified forms of these used to silence or reduce gene expression in a sequence specific manner.

Suppression effectors, such as antisense nucleic acids can be DNA or RNA, can typically be directed to coding sequence however suppression effectors can be targeted to coding sequence and can also be targetted to 5' and/or 3' untranslated regions and/or introns and/or control regions and/or sequences adjacent to a gene or to any combination of such regions of a gene. Antisense nucleic acids including both coding and non-coding sequence can be used if required to help to optimise suppression. Binding of the suppression effector(s) prevents or lowers functional expression of the endogenous gene.

Generally the term 'functional expression' means the expression of a gene product able to function in a

manner equivalent to or better than a wild type product. In the case of a mutant gene or predisposing gene 'functional expression' means the expression of a gene product whose presence gives rise to a deleterious effect or predisposes to a deleterious effect. By deleterious effect is meant giving rise to or predisposing to disease pathology or altering the effect(s) and/or efficiency of an administered compound.

In a particular embodiment of the invention the strategy further employs ribozymes which can be designed to elicit cleavage of target RNAs. The strategy further employs nucleotides which form triple helix DNA. The strategy can employ peptide nucleic acids for suppression. Nucleic acids for antisense, ribozymes, triple helix and peptide nucelic acids may be modified to increase stability, binding efficiencies and uptake (see prior art). Nucleic acids can be incorporated into a vector. Vectors include naked DNA, DNA plasmid vectors, RNA or DNA virus vectors, lipids, polymers or other derivatives and compounds to aid gene delivery and expression.

The invention further provides the use of antisense nucleotides, ribozymes, PNAs, triple helix nucleotides or other suppression effectors alone or in a vector or vectors, wherein the nucleic acids are able to bind specifically or partially specifically to coding sequences of a gene to prevent or reduce the functional expression thereof, in the preparation of a medicament for the treatment of an autosomal dominant or polygenic disease or to increase the utility and/or action of an administered compound.

In a further embodiment of the invention target

sequences for suppression can include non-coding sequences of the gene.

According to the present invention there is provided a strategy for suppressing specifically or partially specifically an endogenous gene and (if required) introducing a replacement gene, said strategy comprising the steps of:

1. providing nucleic acids or other suppression effectors able to bind to an endogenous gene, gene transcript or gene product to be suppressed and

2. providing genomic DNA or cDNA (complete or partial) encoding a replacement gene wherein the nucleic acids are unable to bind to equivalent regions in the genomic DNA or cDNA to prevent expression of the replacement gene. The replacement nucleic acids will not be recognised by suppression nucleic acids or will be recognised less effectively than the endogenous gene. The coding sequence of replacement nucleic acids can be altered to prevent or reduce efficiency of suppression. Replacement nucleic acids have modifications in one or more third base (wobble) positions such that replacement nucleic acids still code for the wild type or equivalent amino acids.

In a particular embodiment of the invention there is provided a strategy for gene suppression targeted to coding sequences of the gene to be suppressed. Suppression will be specific or partially specific to one allele, for example, to the allele carrying a deleterious mutation. Suppressors are targeted to coding regions of a gene or to a combination of coding

and non-coding regions of a gene. Suppressors can be targeted to a characteristic of one allele of a gene such that suppression is specific or partially specific to one allele of a gene (PCT/GB97/00574). The invention further provides for use of replacement nucleic acids with altered coding sequences such that replacement nucleic acids will not be recognised (or will be recognised less effectively) by suppression nucleic acids which are targeted specifically or partially specifically to one allele of the gene to be suppressed. Replacement nucleic acids provide the wild type gene product, an equivalent gene product or an improved gene product but are protected completely or partially from suppression effectors targeted to coding sequences.

In a further embodiment of the invention replacement nucleic acids are provided such that replacement nucleic acids will not be recognised by naturally occurring suppressors found to inhibit or reduce gene expression in one or more individuals, animals or plants. The invention provides for use of replacement nucleic acids which have altered sequences targeted by suppressors of the gene such that suppression by naturally occurring suppressors is completely or partially prevented.

In an additional embodiment of the invention there is provided replacement nucleic acids with altered nucleotide sequence in coding regions such that replacement nucleic acids code for a product with one or more altered amino acids. Replacement nucleic acids provide a gene product which is equivalent to or improved compared with the naturally occurring endogenous wild type gene product.

In an additional embodiment of the invention there is provided a strategy to suppress a gene where the gene transcript or gene product interferes with the action of an administered compound.

The invention further provides the use of a vector or vectors containing suppression effectors in the form of nucleic acids, said nucleic acids being directed towards coding sequences or combinations of coding and non-coding sequences of the target gene and vector(s) containing genomic DNA or cDNA encoding a replacement gene sequence to which nucleic acids for suppression are unable to bind (or bind less efficiently), in the preparation of a combined medicament for the treatment of an autosomal dominant or polygenic disease. Nucleic acids for-suppression or replacement gene nucleic acids may be provided in the same vector or in separate vectors. Nucleic acids for suppression or replacement gene nucleic acids may be provided as a combination of nucleic acids alone or in vectors.

The invention further provides a method of treatment for a disease caused by an endogenous mutant gene, said method comprising sequential or concomitant introduction of

(a) nucleic acids to the coding regions of a gene to be suppressed and/or nucleic acids to coding regions and any combination of 5′ and/or 3′ untranslated regions, intronic regions, control regions or regions adjacent to a gene to be suppressed

(b) replacement nucleic acids with sequences which allow the replacement gene to be expressed.

The nucleic acid for gene suppression can be administered before, after or at the same time as the replacement gene is administered.

The invention further provides a kit for use in the treatment of a disease caused by a deleterious mutation in a gene, the kit comprising nucleic acids for suppression able to bind to the gene to be suppressed and if required a replacement nucleic acid to replace the mutant gene having sequence which allows it to be expressed and completely or partially escape suppression.

Nucleotides can be administered as naked DNA or RNA. Nucleotides can be delivered in vectors. Naked nucleic acids or nucleic acids in vectors can be delivered with lipids or other derivatives which aid gene delivery. Nucleotides may be modified to render them more stable, for example, resistant to cellular nucleases while still supporting RNaseH mediated degradation of RNA or with increased binding efficiencies (see prior art). Antibodies or peptides can be generated to target the protein product from the gene to be suppressed.

The strategy described herein has applications for alleviating autosomal dominant diseases. Complete silencing of a disease allele may be difficult to achieve using antisense, PNA, ribozyme and triple helix approaches or any combination of gene silencing approaches. However small quantities of mutant product may be tolerated in some autosomal dominant disorders. In others a significant reduction in the proportion of mutant to normal product may result in an amelioration of disease symptoms. Hence this invention may be applied to any autosomal dominantly or polygenically inherited disease in man where the molecular basis of

the disease has been established or is partially understood. This strategy will enable the same therapy to be used to treat a range of different disease mutations within the same gene. The development of such approaches will be important to future therapies for autosomal dominant and polygenic diseases, the key to a general strategy being that it circumvents the need for a specific therapy for every mutation causing or predisposing to a disease. This is particularly relevant in some disorders, for example, rhodopsin linked autosomal dominant RP, in which to date about one hundred different mutations in the rhodopsin gene have been observed in adRP patients. Likewise hundreds of mutations have been identified in the human type I Collagen 1A1 and 1A2 genes in autosomal dominant osteogenesis imperfecta. Costs of developing therapies for each mutation are prohibitive at present. Inventions such as this using a general approach for therapy will be required. General approaches may be targeted to the primary defect as is the case with this invention or to secondary effects such as apoptosis.

This invention may be applied in gene therapy approaches for biologically important polygenic disorders affecting large proportions of the world's populations such as age related macular degeneration, glaucoma, manic depression, cancers having a familial component and indeed many others. Polygenic diseases require inheritance of more than one mutation (component) to give rise to the disease state. Notably an amelioration in disease symptoms may require reduction in the presence of only one of these components, that is, suppression of one genotype which, together with others leads to the disease phenotype, may be sufficient to prevent or ameliorate symptoms of the disease. In some cases suppression of more than

one component may be required to improve disease symptoms. This invention may be applied in possible future interventive therapies for common polygenic diseases to suppress a particular genotype(s) using suppression and when necessary replacement steps.

The present invention is exemplified using four genes: human rhodopsin, mouse rhodopsin, human peripherin and human collagen 1A2. The first of these genes are retinal specific. In contrast, collagen 1A2 is expressed in a range of tissues including skin and bone. While these four genes have been used as examples there is no reason why the invention could not be deployed in the suppression of many other genes in which mutations cause or predispose to a deleterious effect. Many examples of mutant genes which give rise to disease phenotypes are available from the prior art - these genes all represent targets for the invention. The present invention is exemplified using hammerhead ribozymes with antisense arms to elicit RNA cleavage. There is no reason why other suppression effectors directed towards genes, gene transcripts or gene products could not be used to achieve gene suppression. Many examples from the prior art detailing use of suppression effectors inter alia antisense RNA/DNA, triple helix, PNAs and peptides to achieve suppression of gene expression are reported (see prior art). The present invention is exemplified using hammerhead ribozymes with antisense arms to elicit sequence specific cleavage of transcripts from genes implicated in dominant disorders and non-cleavage of transcripts from replacement genes containing sequence modifications in wobble positions such that the replacement gene still codes for wild type protein. The present invention is exemplified using suppression effectors targeting sites in coding regions of the

human and mouse rhodopsin, human peripherin and human collagen 1A2 genes. Target sites which include sequences from transcribed but untranslated regions of genes, introns, regions involved in the control of gene expression, regions adjacent to the gene or any combination of these could be used to achieve gene suppression. Multiple suppression effectors, for example, shotgun ribozymes could be used to optimise efficiency of suppression when necessary. Additionally, when required expression of a modified replacement gene such that the replacement gene product is altered from the wild type product such that it provides a beneficial effect may be used to provide gene product.

MATERIALS and METHODS

Cloning vectors

cDNA templates and ribozymes were cloned into commercial expression vectors (pCDNA3, pZeoSV or pBluescript) which enable expression in a test tube from T7, T3 or SP6 promoters or expression in mammalian cells from CMV or SV40 promoters. Inserts were placed into the multiple cloning site (MCS) of these vectors typically at or near the terminal ends of the MCS to delete most of the MCS and thereby prevent any possible problems with efficiency of expression subsequent to cloning.

Sequencing protocols

Clones containing template cDNAs and ribozymes were sequenced by ABI automated sequencing machinery using

standard protocols.

Expression of RNAs

RNA was obtained from clones *in vitro* using a commercially available Ribomax expression system (Promega) and standard protocols. RNA purifications were undertaken using the Bio-101 RNA purification kit or a solution of 0.3M sodium acetate and 0.2% SDS after isolation from polyacrylamide gels. Cleavage reactions were performed using standard protocols with varying $MgCl_2$ concentrations (0-15mM) at 37°C, typically for 3 hours. Time points were performed at the predetermined optimal $MgCl_2$ concentrations for up to 5 hours. Radioactively labelled RNA products were obtained by incorporating a-P32 rUTP (Amersham) in the expression reactions (Gaughan et al. 1995). Labelled RNA products were run on polyacrylamide gels before cleavage reactions were undertaken for the purpose of RNA purification and subsequent to cleavage reactions to establish if RNA cleavage had been achieved. Cleavage reactions were undertaken with 5mM Tris-HCl pH8.0 and varying concentrations of $MgCl_2$ at 37°C.

RNA secondary structures

Predictions of the secondary structures of human and mouse rhodopsin, human peripherin and human collagen 1A2 mRNAs were obtained using the RNAPlotFold program. Ribozymes and antisense were designed to target areas of the RNA that were predicted to be accessible to suppression effectors, for example open loop structures. The integrity of open loop structures was evaluated from the 10 most probable RNA structures. Additionally, predicted RNA structures for truncated RNA products were generated and the integrity of open

loops between full length and truncated RNAs compared.

TEMPLATES and RIBOZYMES

Human Rhodopsin

Template cDNA

The human rhodopsin cDNA was cloned into the HindIII and EcoRI sites of the MCS of pCDNA3 in a 5′ to 3′ orientation allowing subsequent expression of RNA from the T7 or CMV promoter in the vector. The full length 5′UTR sequence was inserted into this clone using primer driven PCR mutagenesis and a HindIII (in pCDNA3) to BstEII (in the coding sequence of the human rhodopsin cDNA) DNA fragment (Sequence 1).

cDNA with altered sequence at a wobble position

The human rhodopsin hybrid cDNA with a single base alteration, a C-->G change (at position 477) was introduced into human rhodopsin cDNA, using a HindIII to BstEII PCR cassette, by primer directed PCR mutagenesis. This sequence change occurs at a silent position - it does not give rise to an amino acid substitution - however it eliminates the ribozyme cleavage site (GUX -->GUG). The hybrid rhodopsin was cloned into pCDNA3 in a 5′ to 3′ orientation allowing subsequent expression of RNA from the T7 or CMV promoter in the vector (Sequence 2).

Rhodopsin cDNA carrying a Pro23Leu adRP mutation

A human rhodopsin adRP mutation, a C-->T change (at codon 23) was introduced into human rhodopsin cDNA, using a HindIII to BstEII PCR cassette by primer directed PCR mutagenesis. This sequence change results in the substitution of a Proline for a Leucine residue. Additionally the nucleotide change creates a ribozyme

cleavage site (CCC-->CTC). The mutated rhodopsin was cloned into the HindIII and EcoRI sites of pCDNA3 in a 5' to 3' orientation allowing subsequent expression of RNA from the T7 or CMV promoter in the vector (Sequence 3).

Ribozyme constructs

A hammerhead ribozyme (termed Rz10) designed to target a large conserved open loop structure in the RNA from the coding regions of the gene was cloned subsequent to synthesis and annealing into the HindIII and XbaI sites of pCDNA3 again allowing expression of RNA from the T7 or CMV promoter in the vector (Sequence 4). The target site was GUC (the GUX rule) at position 475-477 of the human rhodopsin sequence. A hammerhead ribozyme (termed Rz20) designed to target an open loop structure in RNA from the coding region of a mutant rhodopsin gene with a Pro23Leu mutation was cloned subsequent to synthesis and annealing into the HindIII and XbaI sites of pCDNA3 again allowing expression of RNA from the T7 or CMV promoter in the vector (Sequence 5). The target site was CTC (the NUX rule) at codon 23 of the human rhodopsin sequence (Accession number: K02281).
Antisense flanks are underlined.

Rz10: GGACGGTCTGATGAGTCCGTGAGGACGAAACGTAGAG

Rz20: TACTCGAACTGATGAGTCCGTGAGGACGAAAGGCTGC

Mouse rhodopsin

Template cDNA

The full length mouse rhodopsin cDNA was cloned into the EcoRI sites of the MCS of pCDNA3 in a 5' to 3' orientation allowing subsequent expression of RNA from the T7 or CMV promoter in the vector (Sequence 6).

cDNA with altered sequence at a wobble position

The mouse rhodopsin hybrid cDNA with a single base alteration, a T-->C change (at position 1460) was introduced into mouse rhodopsin cDNA, using a HindIII to Eco47III PCR cassette, by primer directed PCR mutagenesis. This sequence change occurs at a silent position - it does not give rise to an amino acid substitution - however it eliminates the ribozyme cleavage site (TTT-->TCT). The hybrid rhodopsin was cloned into pCDNA3 in a 5′ to 3′ orientation allowing subsequent expression of RNA from the T7 or CMV promoter in the vector (Sequence 7).

Ribozyme constructs

A hammerhead ribozyme (termed Rz33) designed to target a large robust open loop structure in the RNA from the coding regions of the gene was cloned subsequent to synthesis and annealing into the HindIII and XbaI sites of pCDNA3 again allowing expression of RNA from the T7 or CMV promoter in the vector (Sequence 8). The target site was TTT (the NUX rule) at position 1459-1461 of the mouse rhodopsin sequence. (Accession number: M55171). Antisense flanks are underlined.

Rz33: GGCACATCTGATGAGTCCGTGAGGACGAAAAAATTGG

Human peripherin

Template cDNA

The full length human peripherin cDNA was cloned into the EcoRI sites of the MCS of pCDNA3 in a 5′ to 3′ orientation allowing subsequent expression of RNA from the T7 or CMV promoter in the vector (Sequence 9).

DNAs with altered sequence at a wobble position

A human peripherin hybrid DNA with a single base alteration, a A-->G change (at position 257) was introduced into human peripherin DNA by primer directed

PCR mutagenesis (forward 257 mutation primer - 5'CATGGCGCTGCTGAAAGTCA3'- the reverse 257 primer was complementary to the forward primer). This sequence change occurs at a silent position - it does not give rise to an amino acid substitution - however it eliminates the ribozyme cleavage site (CTA-->CTG)(Sequence 10). A second human peripherin hybrid DNA with a single base alteration, a A-->G change (at position 359) was introduced into human peripherin DNA, again by primer directed PCR mutagenesis (forward 359 mutation primer - 5'CATCTTCAGCCTGGGACTGT3' - the reverse 359 primer was complementary to the forward primer) (Sequence 11). Similarly this sequence change occurs at a silent position - it does not give rise to an amino acid substitution - however again it eliminates the ribozyme cleavage site (CTA-->CTG). The ribozyme cleavage sites at 255-257 and 357-359 occur at different open loop structures as predicted by the RNAPlotFold program. Hybrid peripherin DNAs included the T7 promoter sequence allowing subsequent expression of RNA.

Ribozyme constructs

Hammerhead ribozymes (termed Rz30 and Rz31), designed to target robust open loop structures in the RNA from the coding regions of the gene, were cloned subsequent to synthesis and annealing into the HindIII and XbaI sites of pCDNA3 again allowing expression of RNA from the T7 or CMV promoter in the vector (Sequences 12+13). The target sites were both CTA (the NUX rule) at positions 255-257 and 357-359 respectively of the human peripherin sequence. (Accession number: M73531). Antisense flanks are underlined.

Rz30: ACTTTCAGCTGATGAGTCCGTGAGGACGAAAGCGCCA

Rz31: ACAGTCCCTGATGAGTCCGTGAGGACGAAAGGCTGAA

Human Type I Collagen - COL1A2

Template cDNA

A human type I collagen 1A2 cDNA was obtained from the ATCC (Accession No: Y00724). A naturally occurring polymorphism has previously been found in collagen 1A2 at positions 907 of the gene involving a T-->A nucleotide change (Filie et al. 1993). The polymorphism occurs in a predicted open loop structure of human collagen 1A2 RNA. Polymorphic variants of human collagen 1A2 were generated by PCR directed mutagenesis, using a HindIII to XbaI PCR cassette. Resulting clones contained the following polymorphism : collagen 1A2 (A) has a A nucleotide at position 907 (Sequence 14). In contrast human collagen 1A2 (B) has a T nucleotide at position 907 (Sequence 15). In collagen 1A2 (B) there is a ribozyme target site, that is a GTC site (906-908), however this cleavage site is lost in collagen 1A2 (A) as the sequence is altered to GAC (906-908), thereby disrupting the ribozyme target site.

Ribozyme constructs

A hammerhead ribozyme (termed Rz907) was designed to target a predicted open loop structure in the RNA from the coding region of the polymorphic variant of the human collagen 1A2 gene. Rz907 oligonucleotide primers were synthesised, annealed and cloned into the HindIII and XbaI sites of pCDNA3 again allowing subsequent expression of RNA from the T7 or CMV promoter in the vector (Sequence 16). The target site was a GUX site at position 906-908 of the human type I collagen 1A2 sequence (Accession number: Y00724). Antisense flanks are underlined.

Rz907: CGGCGGCTGATGAGTCCGTGAGGACGAAACCAGCA

FIGURE LEGENDS

Diagram 1
pBR322 was cut with MspI, radioactively labeled and run on a polyacrylamide gel to enable separation of the resulting DNA fragments. The sizes of these fragments are given in diagram 1. This DNA ladder was then used on subsequent polyacrylamide gels (4-8%) to provide an estimate of the size of the RNA products run on the gels. However there is a significant difference in mobility between DNA and RNA depending on the percentage of polyacrylamide and the gel running conditions - hence the marker provides an estimate of size of transcripts.

Figure 1 -
A: Human rhodopsin cDNA was expressed from the T7 promoter to the BstEII site in the coding sequence. Resulting RNA was mixed with Rz10RNA in 15mM magnesium chloride and incubated at 37°C for varying times. Lanes 1-4: Human rhodopsin RNA and R210RNA after incubation at 37°C with 15mM magnesium chloride for 0, 1 2 and 3 hours respectively. Sizes of the expressed RNAs and cleavage products are as expected (Table 1). Complete cleavage of human rhodopsin RNA was obtained with a small residual amount of intact RNA present at 1 hour. Lane 6 is intact unadapted human rhodopsin RNA (BstEII) alone. Lane 5 is unadapted human rhodopsin RNA (FspI) alone and refers to Figure 1B. From top to bottom, human rhodopsin RNA and the two cleavage products from this RNA are highlighted with arrows.
B: The unadapted human rhodopsin cDNA was expressed from the T7 promoter to the FspI site in the coding sequence. The adapted human rhodopsin cDNA was expressed from the T7 promoter to the BstEII site in the coding sequence. Lanes 1-4: Resulting RNAs were

mixed together with Rz10 and 15mM magnesium chloride and incubated at 37°C for varying times (0, 1 , 2 and 3 hours respectively). The smaller unadapted rhodopsin transcripts were cleaved by Rz10 while the larger adapted transcripts were protected from cleavage by Rz10. Cleavage of adapted protected transcripts would have resulted in products of 564bases and 287bases - the 564bases product clearly is not present - the 287bp product is also generated by cleavage of the unadapted human rhodopsin tanscripts and hence is present (FspI). After 3 hours the majority of the unadapted rhodopsin transcripts has been cleaved by Rz10. Lane 5 contains the intact adapted human rhodopsin RNA (BstEII) alone. From top to bottom adapted uncleaved human rhodopsin transcripts, residual unadapted uncleaved human rhodopsin transcripts and the larger of the cleavage products from unadapted human rhodopsin transcripts are highlighted by arrows. The smaller 22bases cleavage product from the unadapted human rhodopsin transcripts has run off the gel.

Figure 2

A: Unadapted and adapted human rhodopsin cDNAs were expressed from the T7 promoter to the AcyI after the coding sequence and the BstEII site in the coding sequence respectively. Sizes of expressed RNAs and cleavage products were as predicted (Table 1). Resulting RNAs were mixed together with Rz10RNA at varying magnesium chloride concentrations and incubated at 37°C for 3 hours. Lane 1: Intact unadapted human rhodopsin RNA (AcyI) alone. Lanes 2-5: Unadapted and adapted human rhodopsin RNAs and Rz10RNA after incubation at 37°C with 0, 5, 10 and 15 mM $MgCl_2$ respectively. Almost complete cleavage of the larger unadapted human rhodopsin RNA was obtained with a small residual amount of intact RNA present at 5 mM $MgCl_2$. In

contrast the adapted human rhodopsin RNA remained intact. From top to bottom, the unadapted and adapted rhodopsin RNAs, and two cleavage products from the unadapted human rhodopsin RNA are highlighted by arrows. Lane 6 is intact adapted human rhodopsin RNA (BstEII) alone.

B: The adapted human rhodopsin cDNA was expressed from the T7 promoter to the BstEII site in the coding sequence. Lanes 1-4: Resulting RNA was mixed together with Rz10 and 0, 5, 10 and 15 mM magnesium chloride and incubated at 37°C for 3 hours respectively. The adapted rhodopsin transcripts were not cleaved by Rz10. Cleavage of adapted transcripts would have resulted in cleavage products of 564bases and 287bases which clearly are not present. Lane 5: intact adapted human rhodopsin RNA (BstEII) alone. Lane 4: RNA is absent - due to a loading error or degradation. The adapted uncleaved human rhodopsin RNA is highlighted by an arrow.

C: Unadapted and adapted human rhodopsin cDNAs were expressed from the T7 promoter to the AcyI after the coding sequence and the BstEII site in the coding sequence respectively. Sizes of expressed RNAs and cleavage products were as predicted (Table 1). Resulting RNAs were mixed together with Rz10RNA at varying magnesium chloride concentrations and incubated at 37°C for 3 hours. Lane 1: DNA ladder as in Diagram 1. Lanes 2-5: Unadapted and adapted human rhodopsin RNAs and Rz10RNA after incubation at 37°C with 0, 5, 10 and 15 mM $MgCl_2$ respectively. Almost complete cleavage of the larger unadapted human rhodopsin RNA was obtained with a small residual amount of intact RNA present at 5 and 10 mM $MgCl_2$. In contrast the adapted human rhodopsin RNA remained intact. Lane 6: Adapted human rhodopsin RNA (BstEII) alone. Lane 7: Unadapted human rhodopsin RNA (AcyI) alone. Lane 8:

DNA ladder as in Diagram 1. From top to bottom, the unadapted and adapted rhodopsin RNAs, and two cleavage products from the unadapted human rhodopsin RNA are highlighted by arrows. Separation of the adapted human rhodopsin RNA (851bases) and the larger of the cleavage products from the unadapted RNA (896bases) is incomplete in this gel (further runnning of the gel would be required to achieve separation) - however the separation of these two RNAs is demonstrated in Figure 2A.

Figure 3

The mutant (Pro23Leu) human rhodopsin cDNA was expressed from the T7 promoter to the BstEII in the coding sequence. Likewise the Rz20 clone was expressed to the XbaI site. Resulting RNAs were mixed together with 10mM magnesium chloride concentrations at 37°C for varying times. Sizes of expressed RNAs and cleavage products were as predicted (Table 1). Lane 1: DNA ladder as in Diagram 1. Lanes 2: Pro23Leu human rhodopsin RNA alone. Lanes 3-7 Pro23Leu human rhodopsin RNAand Rz20RNA after incubation at 37°C with 10 mM $MgCl_2$ for 0mins, 30 mins, 1 hr, 2hrs and 5hrs respectively. Almost complete cleavage of mutant rhodopsin transcripts was obtained with a residual amount of intact RNA left even after 5 hours. Lane 8: DNA ladder as in Diagram 1. From top to bottom, intact mutant rhodopsin RNA and the two cleavage products from the mutant human rhodopsin RNA are highlighted by arrows.

Figure 4

The mutant (Pro23Leu) human rhodopsin cDNA was expressed from the T7 promoter to the BstEII in the coding sequence. Likewise the Rz10 clone was expressed to the XbaI site. Resulting RNAs were mixed together

with 10mM magnesium chloride concentrations at 37°C for varying times. Sizes of expressed RNAs and cleavage products were as predicted (Table 1). Lane 1: DNA ladder as in Diagram 1. Lanes 2: Pro23Leu human rhodopsin RNA alone. Lanes 3-7 Pro23Leu human rhodopsin RNAand Rz10RNA after incubation at 37°C with 10 mM $MgCl_2$ for 0mins, 30 mins, 1 hr, 2hrs and 5hrs respectively. Almost complete cleavage of mutant human rhodopsin RNA was obtained with a residual amount of intact RNA remaining even after 5 hours (Lane 7). Lane 8: DNA ladder as in Diagram 1. From top to bottom, intact mutant rhodopsin RNA and the two cleavage products from the mutant human rhodopsin RNA are highlighted by arrows.

Figure 5 -

The mouse rhodopsin cDNA clone was expressed *in vitro* from the T7 promoter to the Eco47III site in the coding sequence. Likewise the Rz33 clone was expressed to the XbaI site. A: Resulting RNAs were mixed together with 10mM magnesium chloride at 37°C for varying times. Sizes of expressed RNAs and cleavage products were as predicted (Table 1). DNA ladder as in Diagram 1. Lane 1: mouse rhodopsin RNA alone. Lanes 2-5 Mouse rhodopsin RNA and Rz33 RNA after incubation at 37°C with 10 mM $MgCl_2$ at 0, 5, 7.5 and 10 mM $MgCl_2$ respectively for 3 hours. Cleavage of mouse rhodopsin RNA was obtained after addition of divalent ions (Lane 3). Residual uncleaved mouse rhodopsin RNA and the two cleavage products from the mouse rhodopsin RNA are highlighted by arrows. B: The adapted mouse rhodopsin cDNA clone with a base change at position 1460 was expressed *in vitro* from the T7 promoter to the Eco47III site in the coding sequence. Likewise the Rz33 clone was expressed to the XbaI site. Resulting RNAs were mixed together with various magnesium chloride

concentrations at 37°C for 3 hours. Sizes of expressed RNAs and cleavage products were as predicted (Table 1). Lane 1: DNA ladder as in Diagram 1. Lane 2: Adapted mouse rhodopsin RNA alone. Lanes 3-6: Adapted mouse rhodopsin RNA and Rz33 RNA after incubation at 37°C with 0, 5, 7.5 and 10 mMMgCl for 3 hours at 37°C. No cleavage of the adapted mouse rhodopsin RNA was observed. The intact adapted mouse rhodopsin RNA is highlighted by an arrow.

Figure 6

The human peripherin cDNA clone was expressed *in vitro* from the T7 promoter to the BglII site in the coding sequence. Likewise Rz30 (targeting a cleavage site at 255-257) was expressed to the XbaI site. A: Resulting RNAs were mixed together with 10mM magnesium chloride at 37°C for varying times. Lane 1: DNA ladder as in Diagram 1. Lane 2: Intact human peripherin RNA alone. Lanes 3-7: Human peripherin RNA and Rz30 RNA after incubation at 37°C with 10 mM MgCl for 3hrs, 2hrs, 1hr, 30 mins and 0 mins respectively. Lane 8: DNA ladder as in Diagram 1. From top to bottom, intact human peripherin RNA and the two cleavage products from the human peripherin RNA are highlighted by arrows. B: Resulting RNAs were mixed with Rz30 RNA at varying magnesium chloride concentrations and incubated at 37°C for 3hrs. Lane 1: DNA ladder as in Diagram 1. Lanes 2-5: Human peripherin RNA and Rz30 after incubation at 37°C with 10, 7.5, 5 and 0 mM magnesium chloride respectively for 3hrs. Lane 6: Intact human peripherin RNA alone. Sizes of the expressed RNAs and cleavage products are as expected (Table 1). Significant cleavage of human peripherin RNA was obtained with a residual amount of intact RNA present at each $MgCl_2$ concentration. From top to bottom, human peripherin RNA and the two cleavage products from this RNA are

highlighted with arrows. C: The adapted human peripherin DNA with a single base change at position 257 was expressed from the T7 promoter to the AvrII site in the coding sequence. Resulting RNA was mixed with Rz30 at various magnesium cholride concentrations and incubated at 37°C for 3hrs. Lane 1: DNA ladder as in Diagram 1. Lane 2: Intact adapted human peripherin RNA alone. Lanes 3-6: Adapted human peripherin RNA and Rz30 after incubation at 37°C with 0, 5, 7.5 and 10 mM magnesium chloride respectively for 3hrs. Lane 7: DNA ladder as in Diagram 1. D: The unadapted human peripherin cDNA and the adapted human peripherin DNA fragment with a single base change at position 257 were expressed from the T7 promoter to the BglII and AvrII sites respectively in the coding sequence. Resulting RNAs were mixed with Rz30 at various magnesium chloride concentrations and incubated at 37°C for 3hrs. Lane 1: DNA ladder as in Diagram 1. Lane 2: Intact unadapted human peripherin RNA alone. Lane 3: Intact adapted human peripherin RNA alone. Lanes 4-7: Unadapted and adapted human peripherin RNAs and Rz30 after incubation at 37°C with 0, 5, 7.5 and 10 mM magnesium chloride respectively for 3hrs at 37°C. No cleavage of the adapted human peripherin RNA was observed even after 3 hours whereas a significant reduction in the unadapted RNA was observed over the same time frame. Lane 8: DNA ladder as in Diagram 1. From top to bottom, residual unadapted human peripherin RNA, adapted human peripherin RNA and the two cleavage products are highlighted by arrows.

Figure 7

Human peripherin cDNA clone was expressed *in vitro* from the T7 promoter to the BglII site in the coding sequence. Likewise the Rz31 (targeting a cleavage site at 357-359) was expressed to the XbaI site. A:

Resulting RNAs were mixed together with 10mM magnesium chloride at 37°C for varying times. Lane 1: DNA ladder as in Diagram 1. Lanes 2-6: Human peripherin RNA and Rz31 RNA after incubation at 37°C with 10mM MgCl for 3hrs, 2hrs, 1hr, 30mins and 0mins respectively. Increased cleavage of mouse rhodopsin RNA was obtained over time - however significant residual intact RNA remained even after 3 hours (Lane 2). Lane 7: Intact human peripherin RNA alone. Lane 8: DNA ladder as in Diagram 1. From top to bottom, intact human peripherin RNA and the two cleavage products from the human peripherin RNA are highlighted by arrows. B: Resulting RNAs were mixed with Rz31 RNA at varying magnesium chloride concentrations and incubated at 37°C for 3hrs. Lane 1: DNA ladder as in Diagram 1. Lanes 2-5: Human peripherin RNA and Rz31 after incubation at 37°C with 10, 7.5, 5 and 0m M magnesium chloride respectively for 3hrs. Sizes of the expressed RNAs and cleavage products are as expected (Table 1). Significant cleavage of human peripherin RNA was obtained with a residual amount of intact RNA present at each $MgCl_2$ concentration (Lanes 2-4). Lane 6: Intact human peripherin RNA alone. Lane 7: DNA ladder as in Diagram 1. From top to bottim, human peripherin RNA and the two cleavage products from this RNA are highlighted with arrows. C: The adapted human peripherin DNA with a single base change at position 359 was expressed from the T7 promoter to the BglII site in the coding sequence. Resulting RNA was mixed with Rz31 at various magnesium chloride concentrations and incubated at 37°C for 3hrs. Lane 1: DNA ladder as in Diagram 1. Lane 2: Intact adapted human peripherin RNA alone. Lanes 3-6: Adapted human peripherin RNA and RZ31 after incubation at 37°C with 0, 5, 7.5 and 10mM magnesium chloride respectively for 3hrs. No cleavage of the adapted human peripherin RNA was observed even after 3 hours.

Lane 7: DNA ladder as in Diagram 1.

Figure 8

A: The human collagen 1A2 cDNA clones containing the A and T alleles of the polymorphism at position 907 were expressed from the T7 promoter to the MvnI and XbaI sites in the insert and vector respectively. Resulting RNAs were mixed together with Rz907 and various $MgCl_2$ concentrations and incubated at 37°C for 3 hours. Lane 1: intact RNA from the human collagen 1A2 (A) containing the A allele of the 907 polymorphism. Lane 2: intact RNA from the human collagen 1A2 (B) containing the T allele of the 907 polymorphism. Lanes 3-5: Human collagen 1A2 (A) and (B) representing the A and T allele RNAs and Rz907 incubated with 0, 5, and 10 mM $MgCl_2$ at 37°C for 3 hours. RNA transcripts from the T allele containing the 906-908 target site are cleaved by Rz907 upon addition of divalent ions - almost complete cleavage is obtained with a residual amount of transcript from the T allele remaining (Lane 5). In contrast transcripts expressed from the A allele (which are smaller in size to distinguish between the A (MvnI) and T (XbaI) alleles) were not cleaved by Rz907 - no cleavage products were observed. From top to bottom, RNA from the T allele, the A allele and the two cleavage products from the T allele are highlighted by arrows. Lane 6: DNA ladder as in Diagram 1.

B: The human collagen 1A2 cDNA (A) + (B) clones containing the A and T alleles of the polymorphism at 907 were expressed from the T7 promoter to the MvnI and XbaI sites in the insert and vector respectively. Resulting RNAs were mixed together with Rz907 and 10mM magnesium chloride and incubated at 37°C for varying times. Lane 1: DNA ladder as in Diagram 1. Lane 2: intact RNA from the human collagen 1A2 (A) with the A allele of the 907 polymorphism. Lane 3: intact RNA

from the human collagen 1A2 (B) with the T allele of the 907 polymorphism. Lanes 4-9: Human collagen 1A2 A and T allele RNA and Rz907 incubated with10mM $MgCl_2$ at 37°C for 0, 30 mins, 1hour, 2hours, 3 hours and 5hours respectively. RNA transcripts from the T allele containing the 906-908 target site are cleaved by Rz907 - almost complete cleavage is obtained after 5 hours. In contrast transcripts expressed from the A allele (which are smaller in size to distinguish between the A (MvnI) and T (XbaI) alleles) were not cleaved by Rz907 - no cleavage products were observed. From top to bottom, RNA from the T allele, the A allele and the two cleavage products from the T allele are highlighted by arrows.

Sequences

Sequence 1
The human rhodopsin cDNA in pCDNA3.

Sequence 2
The human rhodopsin cDNA in pCDNA3 with a base change at a silent site (477).

Sequence 3
Mutant (Pro23Leu) human rhodopsin cDNA in pCDNA3.

Sequence 4
Rz10 cloned into pCDNA3. Note there is a one base mismatch in one antisense arm of Rz10.

Sequence 5
Rz20 cloned into pCDNA3

Sequence 6
The mouse rhodopsin cDNA in pCDNA3.

Sequence 7
The mouse rhodopsin cDNA in pCDNA3 with a base change at a silent site (1460).

Sequence 8
Rz33 cloned into pCDNA3

Sequence 9
The human peripherin cDNA in pCDNA3.

Sequence 10
The human peripherin DNA fragment with a base change at a silent site (257).

Sequence 11
The human peripherin DNA fragment with a base change at a silent site (359).

Sequence 12
Rz30 cloned into pCDNA3

Sequence 13
Rz31 cloned into pCDNA3

Sequence 14
Collagen 1A2 (A) sequence containing the A polymorphism at position 907. (Note there is an additional polymorphic site at position 902).

Sequence 15
Collagen 1A2 (B) sequence containing the T polymorphism at position 907. (Note there is an additional polymorphic site at position 902).

Sequence 16
Rz907 cloned into pCDNA3

Note:

The sequence quality was not good in the region of the human peripherin 359 silent change - the sequencing primer was too far from the target site to achieve good quality sequence (Sequence 11).

RESULTS

Human and mouse rhodopsin, human peripherin and human collagen 1A2 cDNA clones were expressed *in vitro*. Ribozymes targeting specific sites in the human and mouse rhodopsin, human peipherin and human collagen 1A2 cDNAs were also expressed *in vitro*. cDNA clones were cut with various restriction enzymes resulting in the production of differently sized transcripts after expression. This aided in differentiating between RNAs expressed from unadapted and adapted cDNAs. Restriction enzymes used to cut each clone, sizes of resulting transcripts and predicted sizes of products after cleavage by target ribozymes are given below in Table 1. Exact sizes of expression products may vary by a few bases from that estimated as there may be some ambiguity concerning inter alia the specific base at which transcription starts.

Example 1

A: Human Rhodopsin

The unadapted human rhodopsin cDNA and the human rhodopsin cDNA with a single nucleotide substitution in the coding sequence were cut with BstEII and expressed *in vitro*. The single base change occurs at the third base position or wobble position of the codon (at position 477) and therefore does not alter the amino acid coded by this triplet. The Rz10 clone was cut with XbaI and expressed *in vitro*. Resulting ribozyme and human rhodopsin RNAs were mixed with varying concentrations of $MgCl_2$ to optimise cleavage of template RNA by Rz10. A profile of human rhodopsin RNA cleavage by Rz10 over time is given in Figure 1A. The $MgCl_2$ curve profile used to test if adapted human

rhodopsin transcripts could be cleaved by Rz10 is given in Figure 2B. Unadapted and adapted human rhodopsin cDNAs were cut with FspI and BstEII respectively, expressed and mixed together with Rz10 RNA to test for cleavage (Figure 1B) over time. Likewise, unadapted and adapted human rhodopsin cDNAs were cut with AcyI and BstEII respectively, both were expressed *in vitro* and resulting transcripts mixed with Rz10 RNA at varying $MgCl_2$ concentrations to test for cleavage (Figure 2A, 2C). In all cases expressed RNAs were the predicted size. Similarly in all cases unadapted transcripts were cleaved into products of the predicted size. Cleavage of unadapted human rhodopsin RNA was almost complete - little residual uncleaved RNA remained. In all cases adapted human rhodopsin RNAs with a single base change at a silent site remained intact, that is, it was not cleaved by Rz10. Clearly, transcripts from the unadapted human rhodopsin cDNA are cleaved by Rz10 while transcripts from the adapted replacement gene which has been modified in a manner which exploits the degeneracy of the genetic code are protected from cleavage. It is worth noting that AcyI enzyme cuts after the stop codon and therefore the resulting RNA includes the complete coding sequence of the gene.

B: Human Rhodopsin

Rz20 was cut with XbaI and expressed in vitro. Similarly the rhodopsin cDNA containing a Pro23Leu mutation was cut with BstEII and expressed *in vitro*. Resulting RNAs were mixed and incubated at 37°C with 10mMMgCl_2 for varying times. Rz20 was designed to elicit mutation specific cleavage of transcripts containing a Pro23Leu rhodopsin mutation. All expressed products and cleavage products were the

correct size. Figure 3 demonstrates mutation specific cleavage of the mutant RNA over time incubated at 37°C with 10mM $MgCl_2$. Cleavage of mutant rhodopsin transcripts by Rz10 which targets a ribozyme cleavage site 3' of the site of the Pro23Leu mutation in rhodopsin coding sequence was explored. The mutant rhodopsin cDNA and Rz10 clones were cut with BstEII and XbaI respectively and expressed *in vitro*. Resulting RNAs were mixed and incubated with 10mM $MgCl_2$ for varying times (Figure 4). All expressed products and cleavage products were the correct size. Rz10 cleaved mutant rhodopsin transcripts. Using a replacement gene with a sequence change around the Rz10 cleavage site which is at a wobble position we demonstrated in Example 1A that transcripts from the replacement gene remain intact due to absence of the Rz10 target site (Figures 1B, 2A and 2B). Hence Rz10 could be used to cleave mutant transcripts in a manner independent of the disease mutation itself (that is, using this site) while transcripts from the replacement gene which code for the correct protein would remain intact and therefore could supply the wild type protein.

Example 2

Mouse Rhodopsin

Rz33 was cut with XbaI and expressed *in vitro*. Similarly the mouse rhodopsin cDNA was cut with Eco47III and expressed *in vitro*. Resulting RNAs were mixed and incubated with varying concentrations of $MgCl_2$. All expressed products and cleavage products were the correct size. Figure 5A demonstrates specific cleavage of the mouse rhodopsin RNA over various $MgCl_2$ concentrations incubated at 37°C for 3 hours. Using a replacement gene with a sequence change around the Rz33 cleavage site (TTT-->TCT) which is at a wobble position we demonstrated that transcripts from the replacement

gene remain intact due to absence of the Rz33 target site (Figures 5B). Hence Rz33 could be used to cleave mutant transcripts in a manner independent of the disease mutation itself (that is, using this site) while transcripts from the replacement gene which code for the correct protein would remain intact and therefore could supply the wild type protein.

Example 3

Human Peripherin

The unadapted human peripherin cDNA and two human peripherin DNA fragments generated by PCR mutagenesis with a single nucleotide substitution in the coding sequence were cut with BglII and AvrII respectively and expressed *in vitro*. The single base changes in the adapted DNAs occur at third base positions or wobble positions of the codon (at position 257 and 359) and therefore do not alter the amino acid coded by these triplets. The Rz30 and Rz31 clones were cut with XbaI and expressed *in vitro*. Resulting ribozymes and unadapted human rhodopsin RNAs were mixed with varying concentrations of $MgCl_2$ to optimise cleavage of template RNA by Rz30 and Rz31. Profiles of human perpherin RNA cleavage by Rz30 over various $MgCl_2$ concentrations and over time are given in Figure 6. Similarly profiles of human perpherin RNA cleavage by Rz31 over various $MgCl_2$ concentrations and over time are given in Figure 7. In all cases expressed RNAs were the predicted size. Similarly in all cases unadapted transcripts were cleaved into products of the predicted size. Adapted human rhodopsin RNAs were mixed together with Rz30 and Rz31 RNA over various $MgCl_2$ concentrations to test if adapted human peripherin transcripts could be cleaved by Rz30 and Rz31 (Figures 6 + 7). Expressed RNAs were the predicted size. In all cases adapted human peripherin RNAs with single base changes at silent

sites remained intact, that is, they were not cleaved by Rz30 or Rz31. Clearly, transcripts from the unadapted human peripherin cDNA are cleaved by Rz30 and Rz31 while transcripts from the adapted replacement DNAs which have been modified in a manner which exploits the degeneracy of the genetic code are protected from cleavage.

Example 4

Human Collagen 1A2

Rz907 clones targeting a polymorphic site in human collagen 1A2 sequence was cut with XbaI and expressed *in vitro*. The human collagen 1A2 cDNA clones (A and B) containing two allelic forms of a polymorphism in the coding sequence of the gene at positions 907 were cut with MvnI and XbaI respectively, expressed *in vitro* and RNAs mixed together with Rz907 RNA to test for cleavage of transcripts by this ribozyme. All expressed transcripts were of the predicted sizes. RNAs were mixed with varying concentrations of $MgCl_2$ to optimise cleavage of RNAs by Rz907 (Figure 8). Notably the majority of the RNA transcripts from human collagen 1A2 (B) which has a T nucleotide at position 907 is cleaved by Rz907 (Figure 8). This allelic form of the gene has a ribozyme cleavage site at 906-908. Notably the situation is reversed with transcripts from human collagen 1A2 (A) where in this allelic form of the gene due to the nature of the polymorphism present at position 907 the ribozyme cleavage site has been lost. In contrast to transcripts from human collagen (B), transcripts from human collagen (A) were protected from cleavage by Rz907 due to the alteration in the sequence around the ribozyme cleavage site (Figure 8). Cleavage of collagen 1A2 (B) by Rz907 was efficient which is consistent with 2-D predictions of RNA open loop structures for the polymorphism - in the allele

containing the Rz907 ribozyme cleavage site, the target site is found quite consistently in an open loop structure. This polymorphism found in an open loop structure of the transcript clearly demonstrates the feasibility and utility of using the degeneracy of the genetic code in the suppression of an endogenous gene (either suppressing both alleles or a single allele at a polymorphic site) and restoration of gene expression using a gene which codes for the same protein but has sequence modifications at third base wobble positions which protect the replacement gene from suppression.

TABLE 1

	Restriction Enzyme	RNA Size	Cleavage Products
Example 1			
Human rhodopsin	BstEII	851 bases	287+564 bases
	AcyI	1183 bases	287+896 bases
	FspI	309 bases	287+22
Adapted Human rhodopsin	BstEII	851 bases	
Human rhodopsin Pro-Leu	BstEII	851 bases	170+681 (Rz20)
Human rhodopsin Pro-Leu	BstEII	851 bases	287+564 (Rz10)
Rz10	XbaI	52 bases	
Rz20	XbaI	52 bases	
(Table 1; Sequences 1-5 ; Figures 1-4)			
Example 2			
Mouse rhodopsin	Eco47III	774 bases	400+374
Adapted mouse rhodopsin	Eco47III	774 bases	
Rz33	XbaI	52 bases	
(Table 1; Sequences 6-9; Figure 5)			
Example 3			
Human peripherin	BglII	545 bases	315+230 (Rz30)
Human peripherin	BglII	545 bases	417+128 (Rz31)
Adapted human peripherin	AvrII	414 bases	
Adapted human peripherin	BglII	545 bases	
Rz30	XbaI	52 bases	
Rz31	XbaI	52 bases	
(Table 1; Sequences 10-14; Figures 6+7)			
Example 4			
Human Collagen 1A2 (B) -Rz907	XbaI	888 bases	690+198 bases
Human Collagen 1A2 (A)	MvnI	837 bases	
Rz907	XbaI	52 bases	
(Table 1; Sequences 14-16; Figure 8)			

TABLE 2

A: Rhodopsin mutations tested to assess if the predicted open loop RNA structure containing the Rz10 target site (475-477) remains intact in mutant transcripts.

Rhodpsin mutation	RNA open loop targeted by Rz10
Pro 23 Leu	Intact
Gly 51 Val	Intact
Thr 94 IIe	Intact
Gly 188 Arg	Intact
Met 207 Arg	Intact
IIe del 255	Intact

B: Utilisation of the degeneracy of the genetic code. Ribozyme cleavage sites are underlined

Human rhodopsin

	475-477
Unadapted sequence	TAC GTC ACC GTC CAG
	Val
	475-477
Adapted sequence	TAC GTG ACC GTC CAG
	Val

Mouse rhodopsin

	1459-1461
Unadapted sequence	AAT TTT TAT GTG CCC
	Phe
	1459-1461
Adapted sequence	AAT TTC TAT GTG CCC
	Phe

Human peripherin

	255-257
Unadapted sequence	GCG CTA CTG AAA GTC
	Leu
	255-257
Adapted sequence	GCG CTG CTG AAA GTC
	Leu
	357-359
Unadapted sequence	AGC CTA GGA CTG TTC
	Leu
	357-359
Adapted sequence	AGC CTG GGA CTG TTC
	Leu

Human type I collagen 1A2

	906-908
Sequence (B)	GCT GGT CCC GCC GGT
	Gly

Sequence (A)	906-908 GCT GGA CCC GCC GGT Gly

DISCUSSION

In the examples outlined above, RNA was expressed from cDNAs coding for four different proteins: human and mouse rhodopsin, human peripherin and human type I collagen 1A2. Rhodopsin and peripherin have been used to exemplify the invention for retinopathies such as adRP - suppression effectors have been targeted to the coding sequences of these genes. In the case of the human collagen 1A2 gene a naturally occurring polymorphism has been used to demonstrate the invention and the potential use of the invention for disorders such as OI - however non-polymorphic regions of the collagen 1A2 gene could be used to achieve suppression. The suppression effectors of choice in the invention have been hammerhead ribozymes with antisense flanks to define sequence specificity. Hammerhead ribozymes require NUX cleavage sites in open loop structures of RNA. Notably, other suppression effectors could be utilised in the invention and may lead to a more flexible choice of target sequences for suppression. Transcripts expressed from all four genes have been significantly attacked *in vitro* using suppression effectors directed towards target cleavage sites. In all four examples the ribozymes directed to cleavage sites were successful in cleaving target RNAs in the predicted manner. Antisense complementary to sequences surrounding the cleavage sites was used successfully to elicit binding and cleavage of target RNAs in a sequence specific manner. Additionally, transcripts from replacement genes, modified using the degeneracy of the genetic code so that they code for wild type protein, were protected fully from cleavage by

ribozymes.

The utility of an individual ribozyme designed to target an NUX site in an open loop structure of transcripts from a gene will depend in part on the robust nature of the RNA open loop structure when various deleterious mutations are also present in the transcript. To evaluate this, we analysed RNAPlotFold data for six different adRP causing mutations in the rhodopsin gene. For each of these, the large RNA open loop structure which is targeted by Rz10 was predicted to be maintained in the mutant transcripts (Table 2A). This is clearly demonstrated in example 1B (Figure 3) using a Pro23Leu rhodopsin mutation. Rz10 clearly cleaves the mutant transcript effectively *in vitro*. The Pro23Leu mutation creates a ribozyme cleavage site and can be cleaved *in vitro* by Rz20 a ribozyme specifically targeting this site - however this is not the case for many mutations. In contrast we have shown that the Rz10 ribozyme cleavage site is available for different mutant rhodopsins and could potentially be used to suppress multiple mutations using a suppression and replacement approach.

In some cases lowering RNA levels may lead to a parallel lowering of protein levels however this may not always be the case. In some situations mechanisms may prevent a significant decrease in protein levels despite a substantial decrease in levels of RNA. However in many instances suppression at the RNA level has been shown to be effective (see prior art). In some cases it is thought that ribozymes elicit suppression not only by cleavage of RNA but also by an antisense effect due to the antisense arms of the ribozyme surrounding the catalytic core.

In all examples provided ribozymes were designed to cleave at specific target sites. Target sites for four of the ribozymes utilised were chosen in open loop structues in the coding regions of transcripts from three retinal genes (human and mouse rhodopsin and human perpherin). In all cases sequence specific cleavage was obtained at the target cleavage sites (Figs 1-7). Target sites were chosen in open loop structures to optimise cleavage. Additionally target sites were chosen such that they could be obliterated by single nucleotide changes at third base wobble positions and therefore would code for the same amino acid (Table 2B). In turn this enabled the generation of replacement genes with single nucleotide alterations which code for wild type protein. In all cases tested transcripts from replacement genes were protected from cleavage by ribozymes. Further modifications could be made to replacement genes in wobble positions, for example, to limit the binding ability of the antisense arms flanking the ribozyme catalytic core. The examples provided for rhodopsin and peripherin involve suppression of expression of both disease and wild type alleles of a retinal gene and restoration of the wild type protein using a replacement gene. However, there may be situations where single alleles can be targeted specifically or partially specifically (PCT/GB97/00574).

In one example, human collagen 1A2, Rz907 was used to target a naturally occurring polymorphic site at amino acid 187, (GGA (glycine) --> GGT (glycine), located in an open loop structure from the predicted 2-D conformations of the transcript (Figure 8, Table 2B). The ribozyme Rz907 cleaved transcripts containing the GGT sequence but transcripts with GGA were protected from cleavage. Transcripts from both alleles of

individuals homozygous for the GGT polymorphism could be cleaved by Rz907 whereas in the case of heterozygotes cleavage could be directed to single alleles (in particular to alleles containing deleterious mutations PCT/GB97/00574). In both situations replacement genes could have the sequence GGA and therefore would be protected from cleavage by Rz907. The presence of many such naturally occurring silent polymorphisms highlights that replacement genes could be modified in a similar fashion in wobble positions and should produce in most cases functional wild type protein. Multiple modifications could be made to replacement genes at wobble positions which would augment protection from suppression effectors. For example, in situations where antisense nucleic acids were used for suppression, transcripts from replacement genes with multiple modifications at third base positions would be protected partially or completely from antisense binding.

In all four examples provided transcripts from cDNA clones were cleaved *in vitro* in a sequence specific manner at ribozyme cleavage sites. Additionally one base of the ribozyme cleavage site occurs at a wobble position and moreover can be altered so as to eliminate the cleavage site. Ribozyme cleavage sites in the examples given were destroyed by changing nucleotide sequences so that the consensus sequence for ribozyme cleavage sites was broken. However it may be that in some cases the cleavage site could be destroyed by altering the nucleotide sequence in a manner that alters the 2-D structure of the RNA and destroys the open loop structure targeted by the ribozyme. cDNAs or DNA fragments with altered sequences in the regions targeted by ribozymes were generated. RNAs expressed from these cDNAs or DNA fragments were protected

entirely from cleavage due to the absence of the ribozyme cleavage site for each of the ribozymes tested. Of particular interest is the fact that a single nucleotide alteration can obliterate a ribozyme target site, thereby preventing RNA cleavage. Although ribozymes have been used in the demonstration of the invention, other suppression effectors could be used to achieve gene silencing. Again replacement genes with altered sequences (at third base wobble positions) could be generated so that they are protected partially or completely from gene silencing and provide the wild type (or beneficial) gene product.

As highlighted before in the text, using the invention the same method of suppression (targeting coding sequences of a gene) and where necessary gene replacement (using a replacement gene with a sequence modified in third base positions to restore gene expression) may be used as a therapeutic approach for many different mutations within a given gene. Given the continuing elucidation of the molecular pathogenesis of dominant and polygenic diseases the number of targets for this invention is rapidly increasing.

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CLAIMS

1. A strategy for suppressing or partially suppressing an endogenous gene and replacing the suppressed gene sequence with a nucleic acid sequence which differs from the endogenous gene and wherein the suppressing agent(s) comprises at least one suppressor from the group comprising antisense nucleic acid, peptide nucleic acids, DNA capable of forming triple helix or ribozymes targeted to the endogenous gene or gene transcripts and wherein the replacement nucleic acid sequence encodes at least part of a gene product and is not suppressed by suppression agent(s) or is suppressed less efficiently by suppression agent(s) and wherein the replacement nucleic acid sequence comprises amino acid codons which encode at least part of the gene product, and have modifications at wobble site(s) such that replacement nucleic acids still code for the wild type or equivalent amino acids.

2. A medicament comprising either one or both of a gene suppressing agent and a nucleic acid encoding at least part of a replacement gene product for use in a strategy as claimed in Claim 1.

3. A medicament comprising a nucleic acid sequence encoding at least part of a gene product wherein the sequence differs from the endogenous gene in wobble sites.

4. A strategy for suppressing or partially suppressing an endogenous gene and introducing a replacement gene said strategy comprising the steps of:

a. providing suppression nucleic acids or other suppression effector(s) able to recognise, bind or cleave an endogenous gene, gene transcript(s) or gene product to be suppressed and

b. providing genomic DNA or cDNA (complete or partial) encoding a replacement gene wherein the suppression nucleic acids are unable to recognise, bind or cleave or able to recognise, bind or cleave less efficiently equivalent regions in the genomic DNA or cDNA to prevent suppression of the replacement gene wherein the coding sequence of replacement nucleic acids has been altered to prevent or reduce efficiency of suppression and wherein replacement nucleic acids have modifications in one or more wobble sites such that replacement nucleic acids still code for the wild type or equivalent amino acids.

5. The use of a strategy as claimed in any of the preceding Claims in the preparation of a medicament for the treatment of an autosomal dominant disease caused by an endogenous target gene wherein the disease is caused by different mutations in the same gene in different patients.

6. The use of:

a. a vector or vectors containing suppression effector(s), said suppression effector(s) being able to recognise, bind or cleave coding sequences of a target endogenous gene and

b. vector(s) containing replacement nucleic acids in the form of genomic DNA, cDNA or

RNA, which contain altered wobble sites such that replacement nucleic acids cannot be recognised, bound or cleaved by suppressor(s) or are recognised, bound or cleaved less efficiently by suppressor(s) which are targeted towards coding sequence of the endogenous gene and which provide the wild type gene product and wherein the difference between said endogenous gene and the replacement gene still enables the expression of the replacement gene,

in the preparation of a medicament for the treatment of an autosomal dominant disease caused by the endogenous gene wherein the disease is caused by different mutations in the same gene in different patients.

7. A use as claimed in Claims 5 or 6 wherein the disease is a polygenic disorder.

8. A use as claimed in Claim 6 or 7 wherein suppressor(s) or replacement gene(s) are administered alone or in vector(s) chosen from DNA plasmid vectors, RNA or DNA viral vectors.

9. A use as claimed in Claim 8 wherein the suppressor(s) or replacement gene(s) are combined with lipids, polymers or other derivatives.

10. A kit for use in the treatment of an autosomal dominant or polygenic disease caused by mutation(s) in a target endogenous gene, the kit comprising at least one suppression effector able to recognise, bind or cleave coding sequence(s) of the endogenous gene to be suppressed and at least

one replacement gene to replace the endogenous gene having modifications to wobble sites such that the replacement gene cannot be recognised, bound or cleaved or can be recognised, bound or cleaved less efficiently by suppressor(s) targeted to coding sequence(s) of the endogenous gene, said replacement nucleic acid sequence providing the wild type gene product, and wherein the difference between said wild type target gene and the replacement gene still enables expression of the replacement gene.

11. A use as claimed as in Claims 1 to 10 wherein the replacement gene is altered from the wild type gene and provides a beneficial effect when compared to the wild type gene.

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Diagram 1

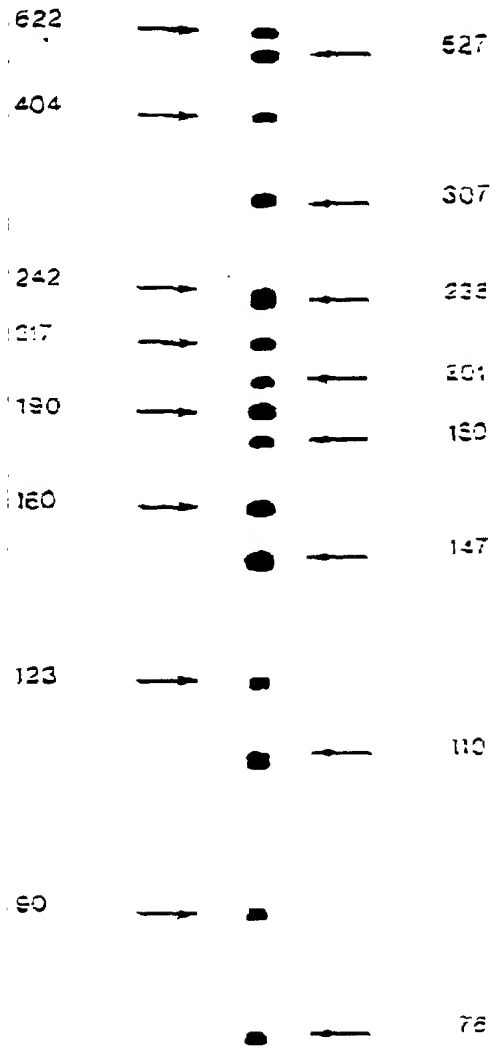

Figure 1 A + B

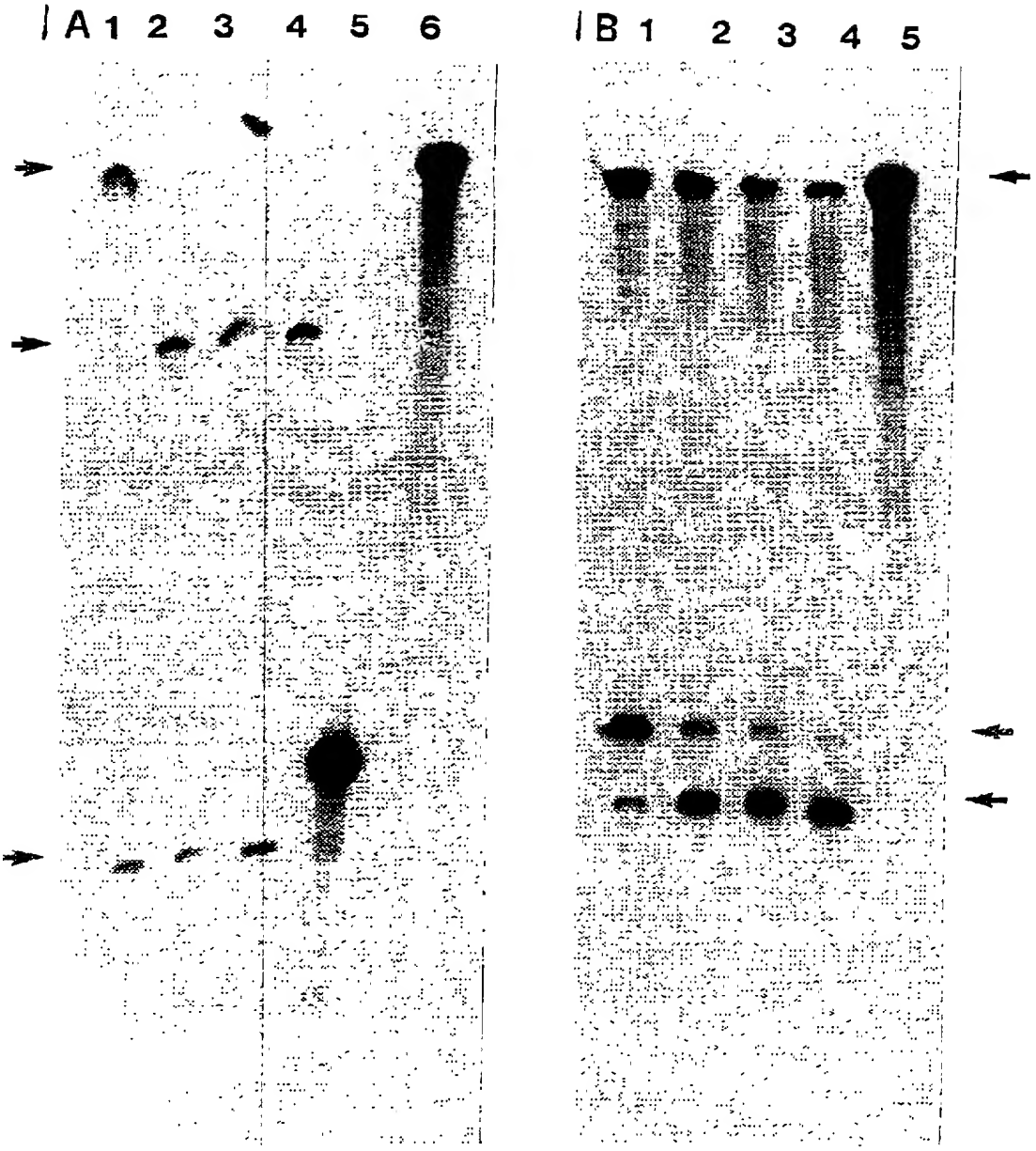

Figure 2 A+B

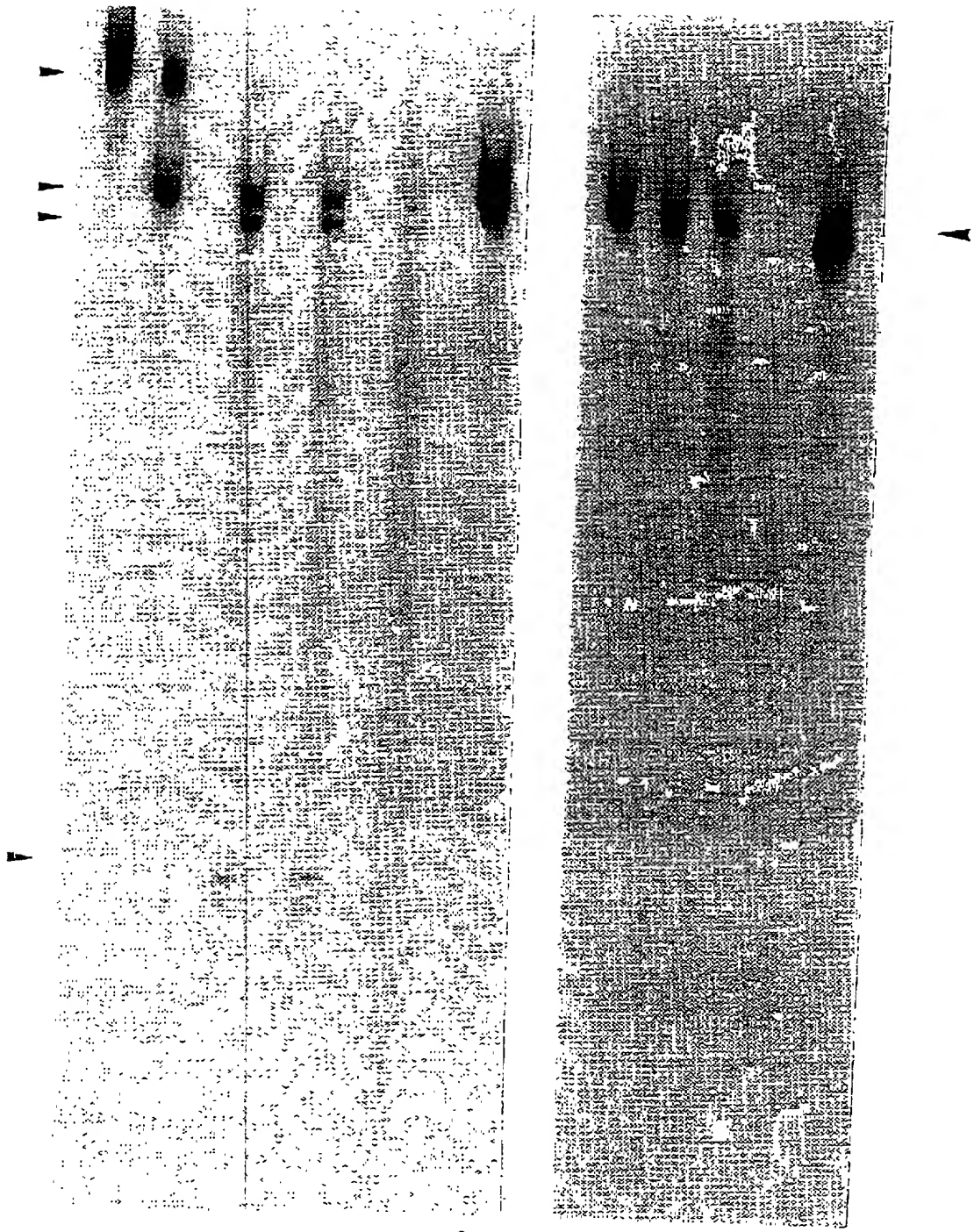

Figure 2c

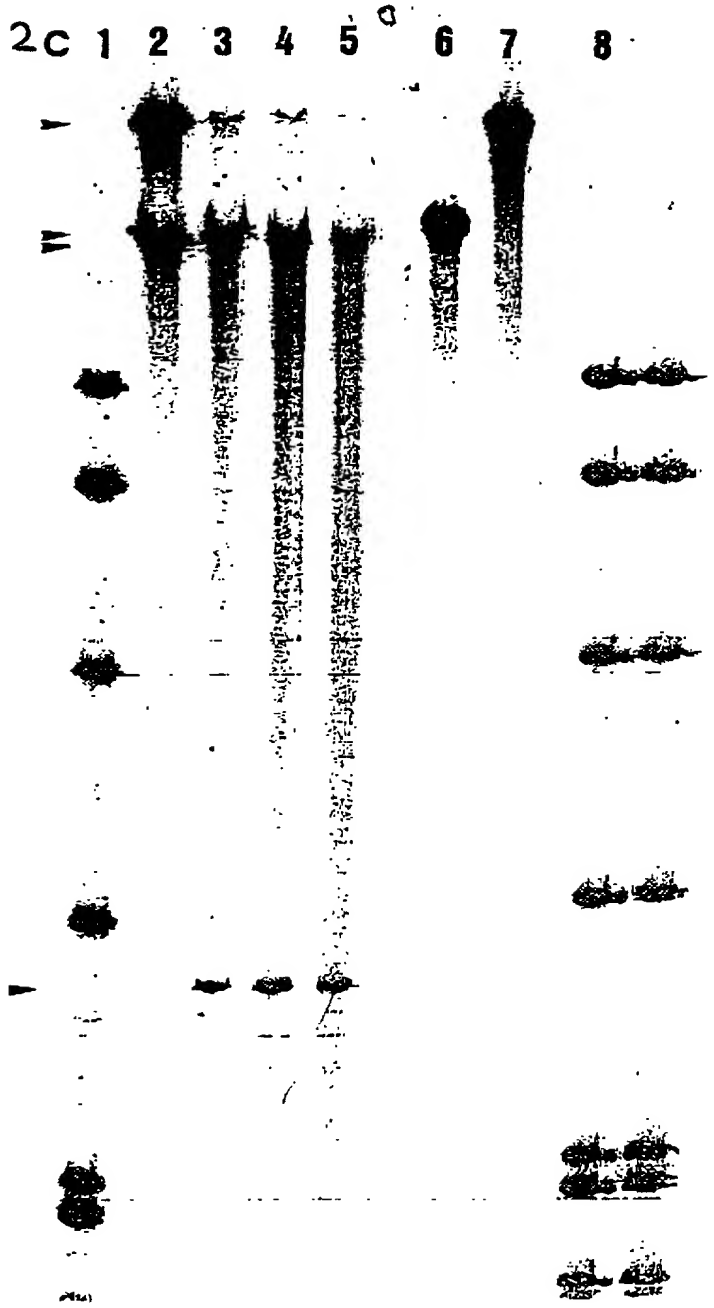

Figure 3

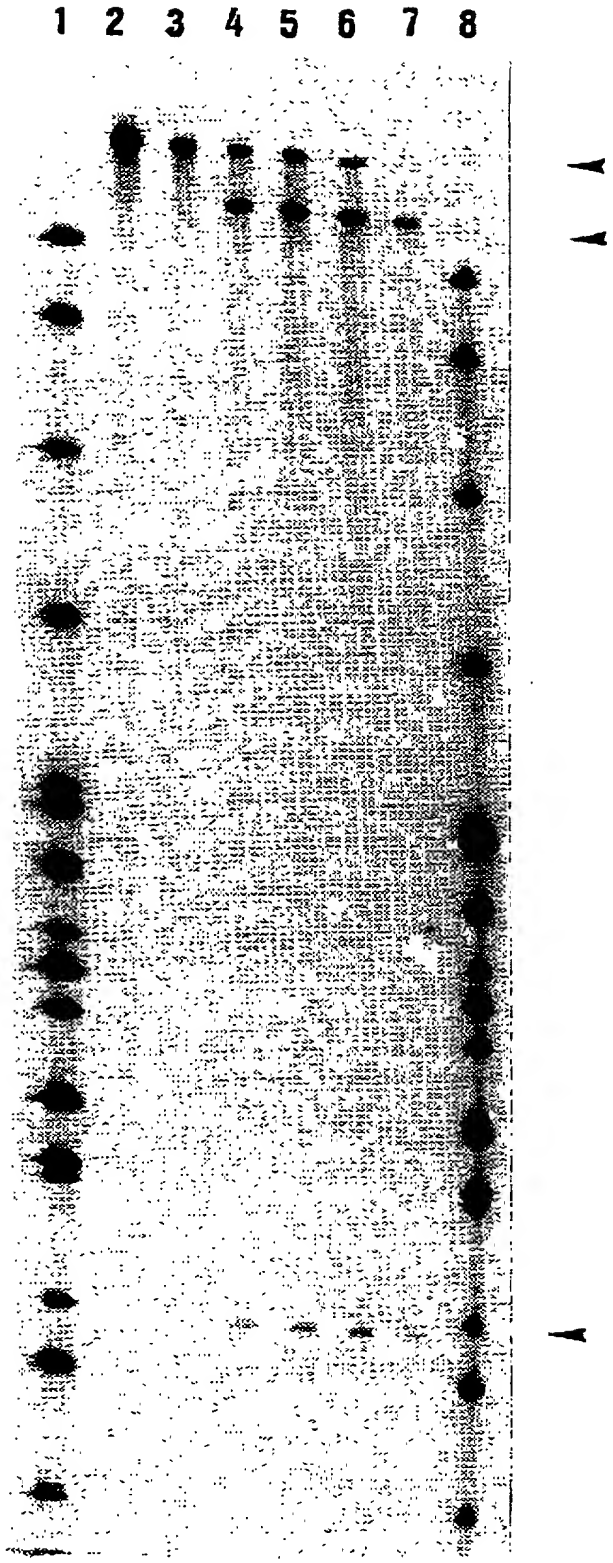

Figure 4

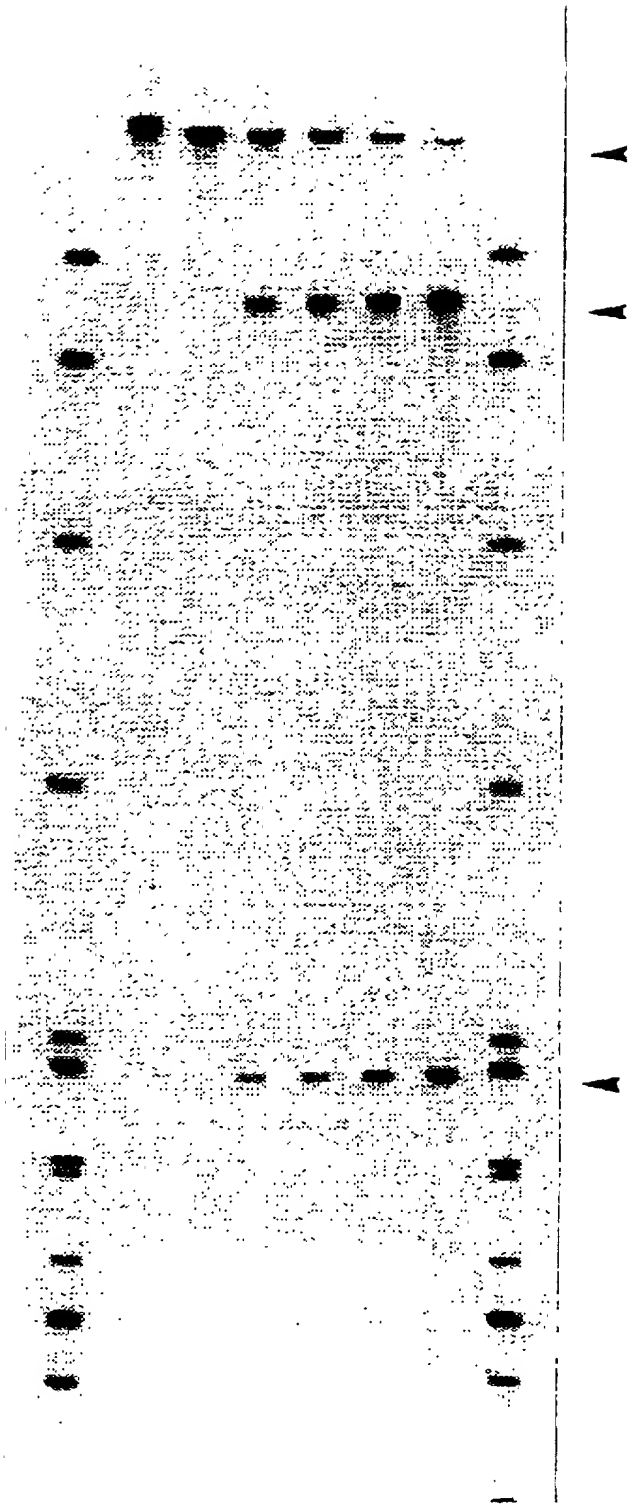

Figure 5 A

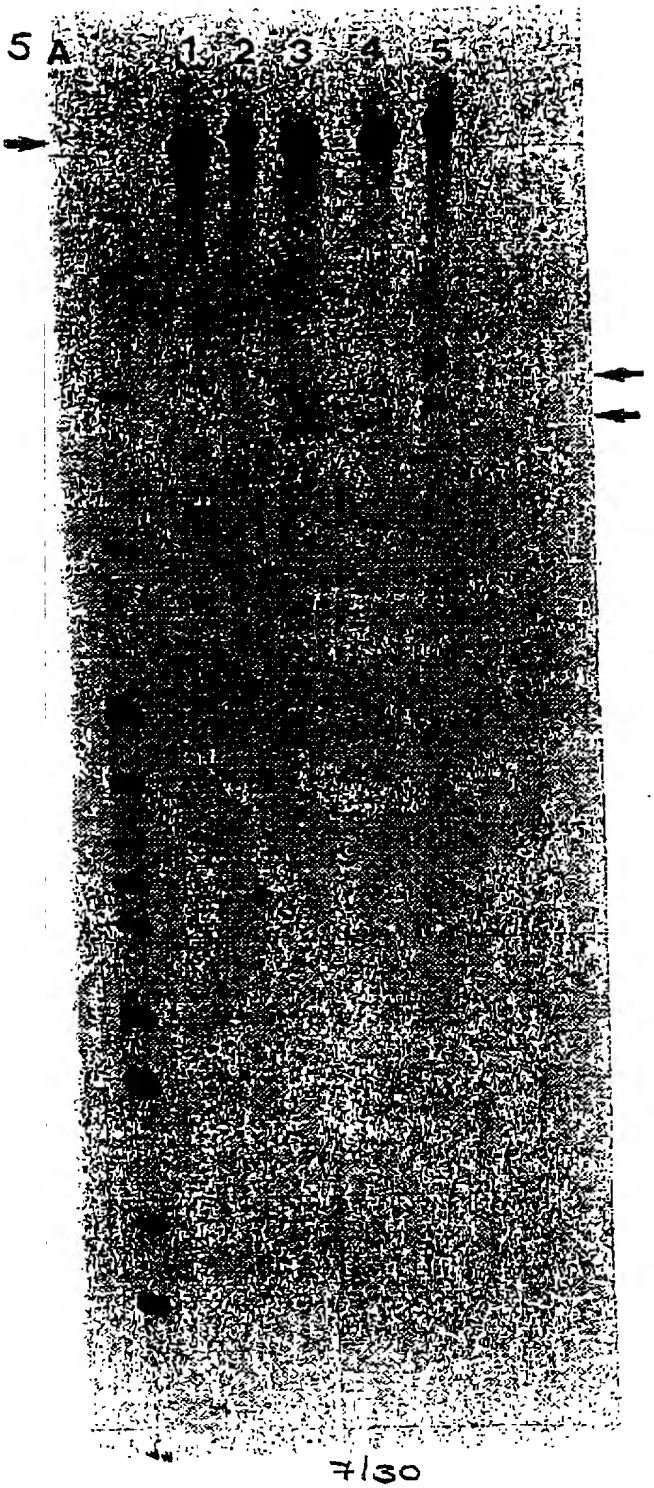

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Figure 5B

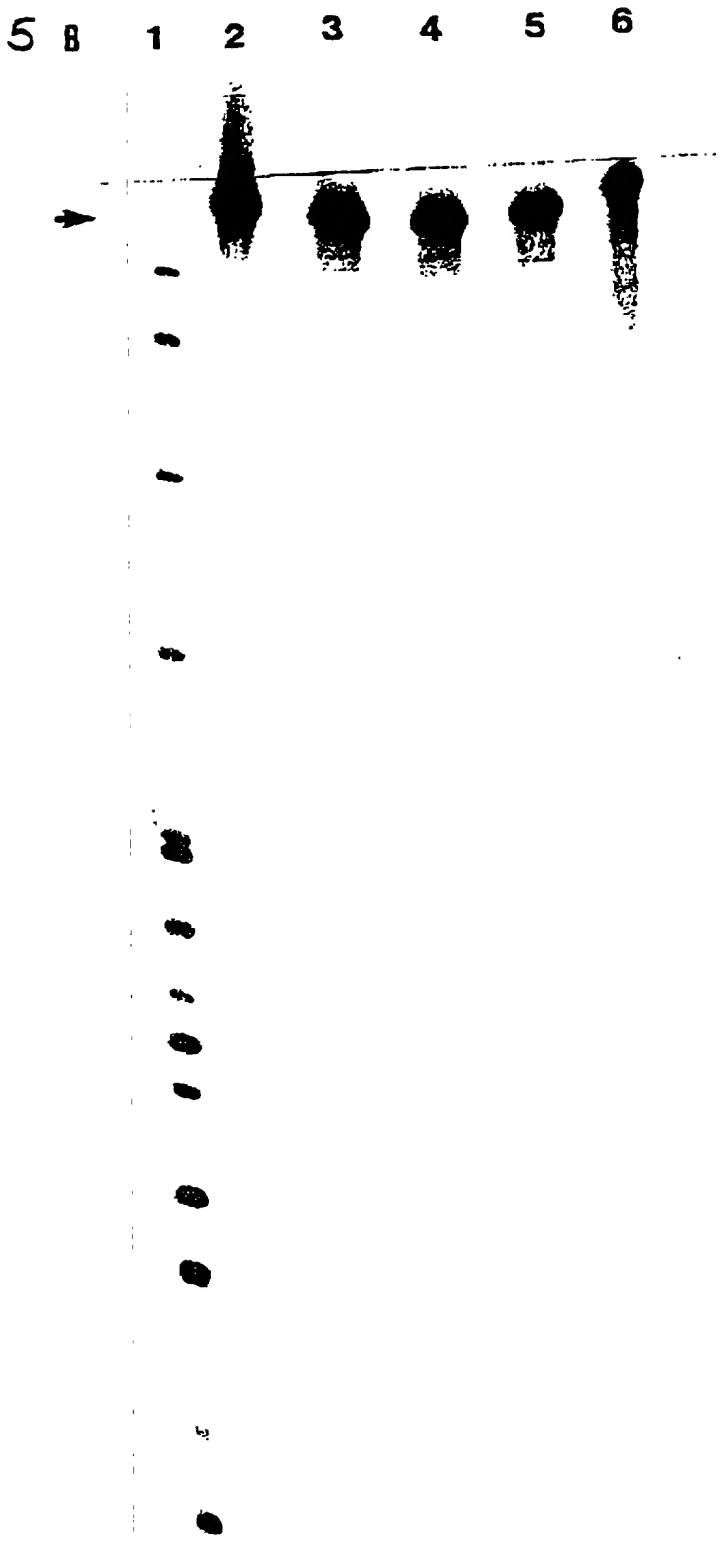

Figure 6 A+B

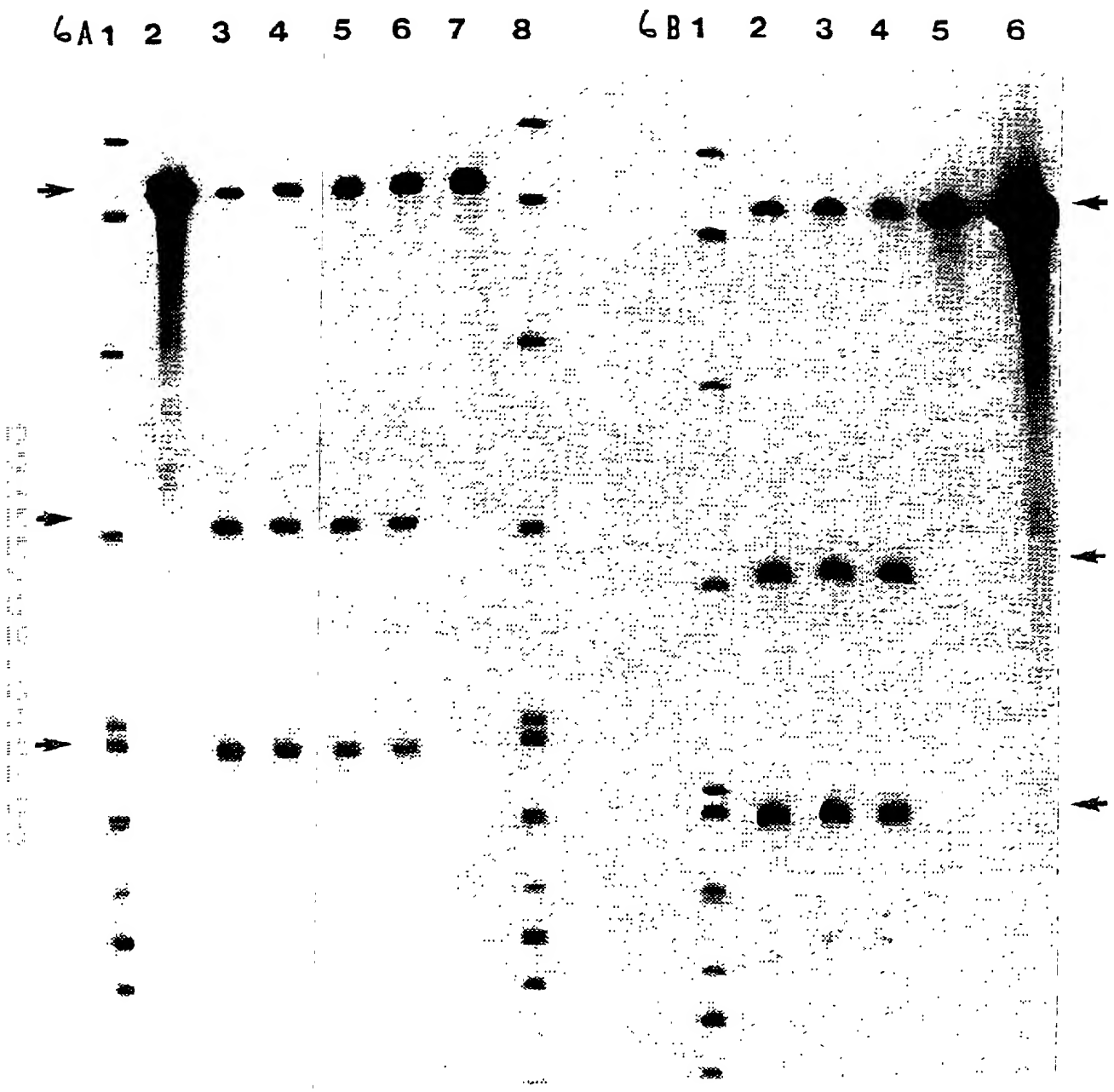

Figure 6C

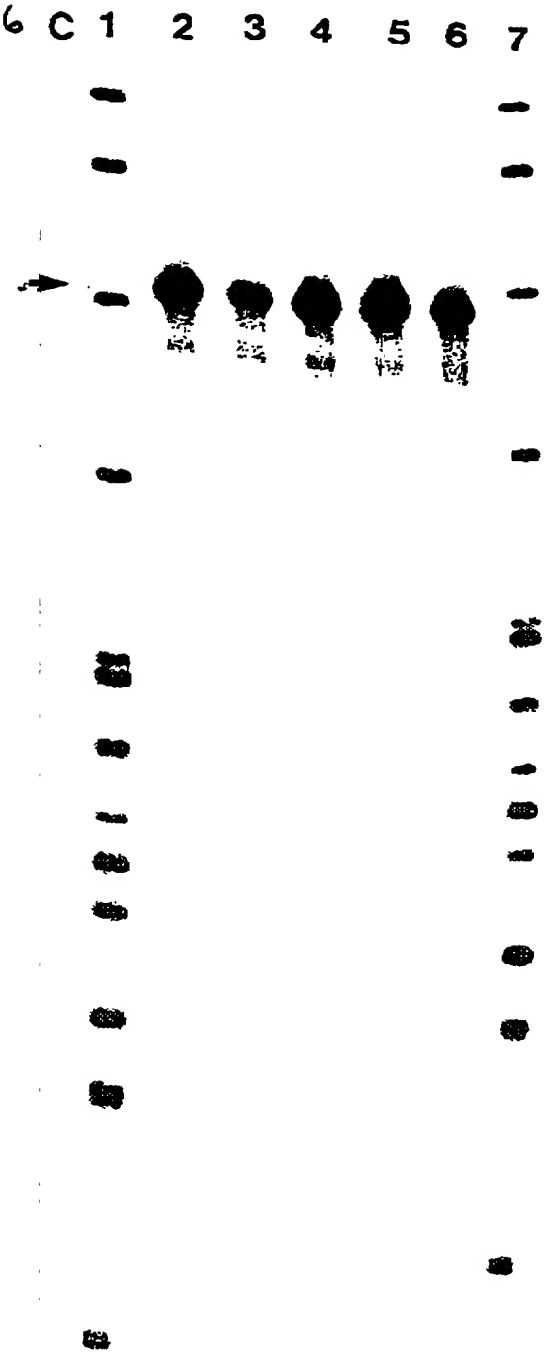

Figure 6D

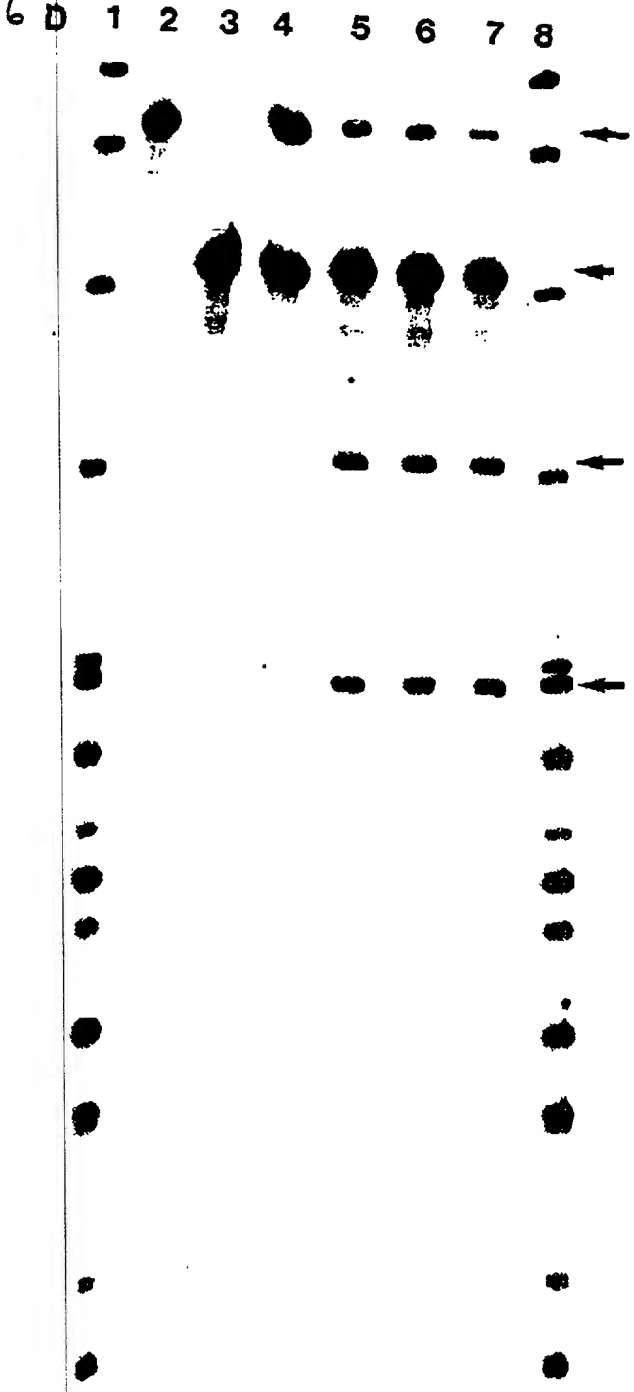

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Figure 7 A + B

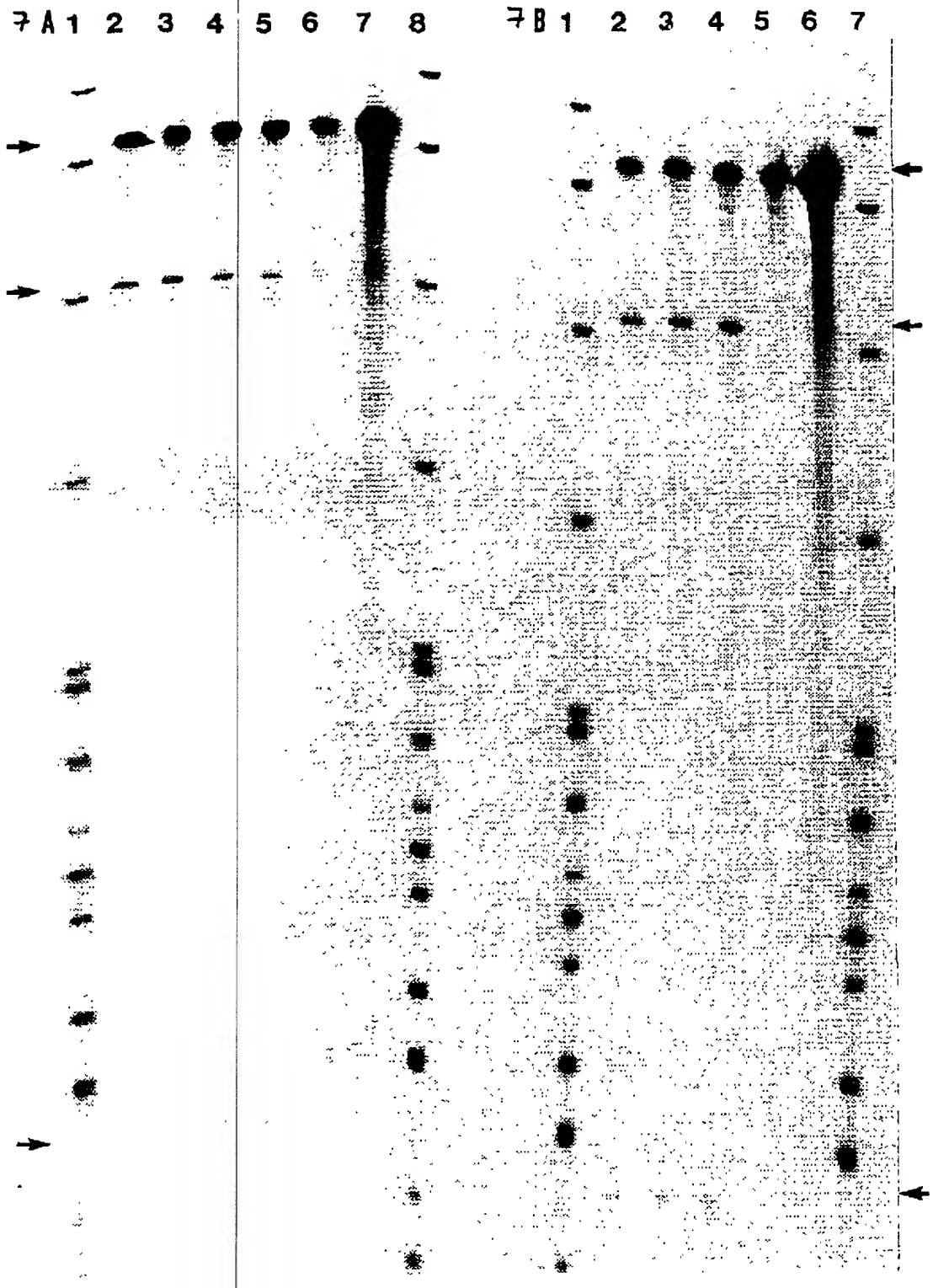


Figure 7C

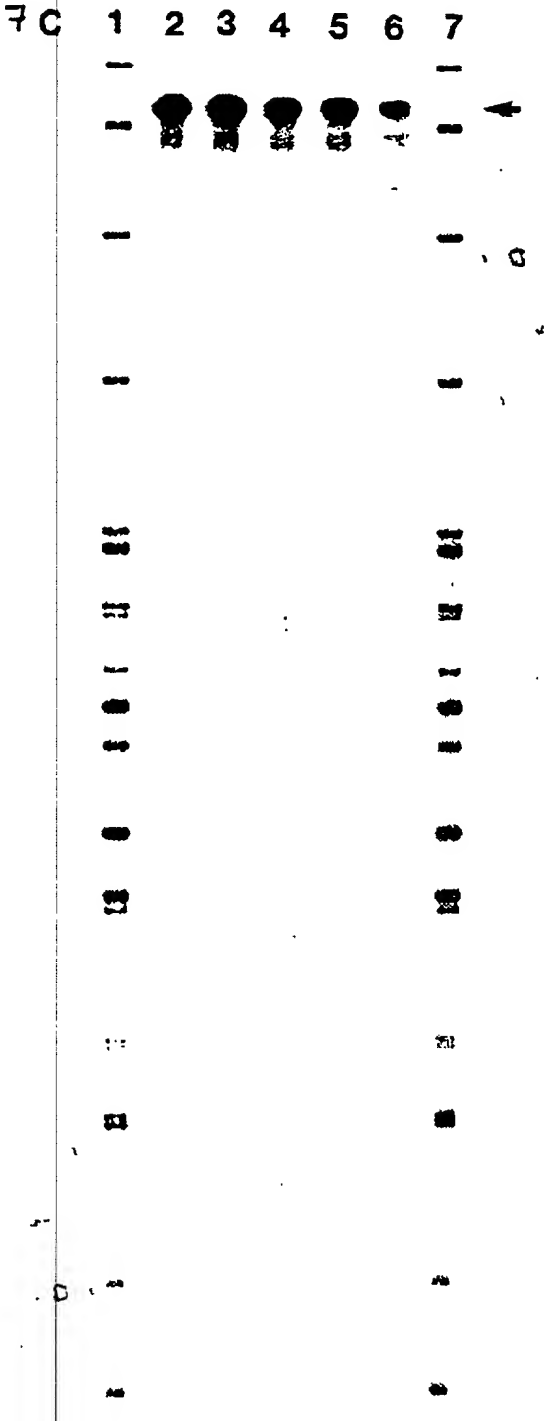
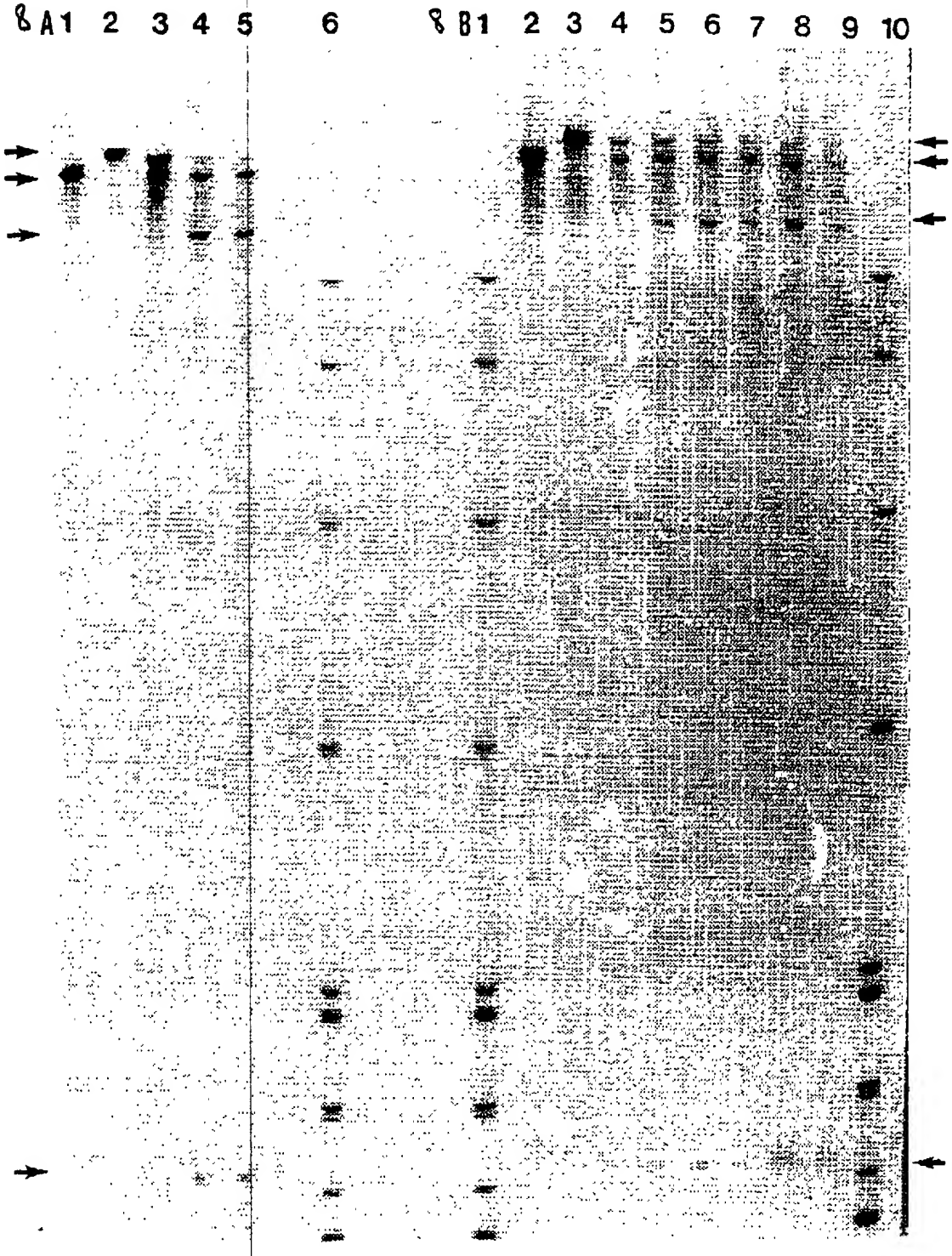
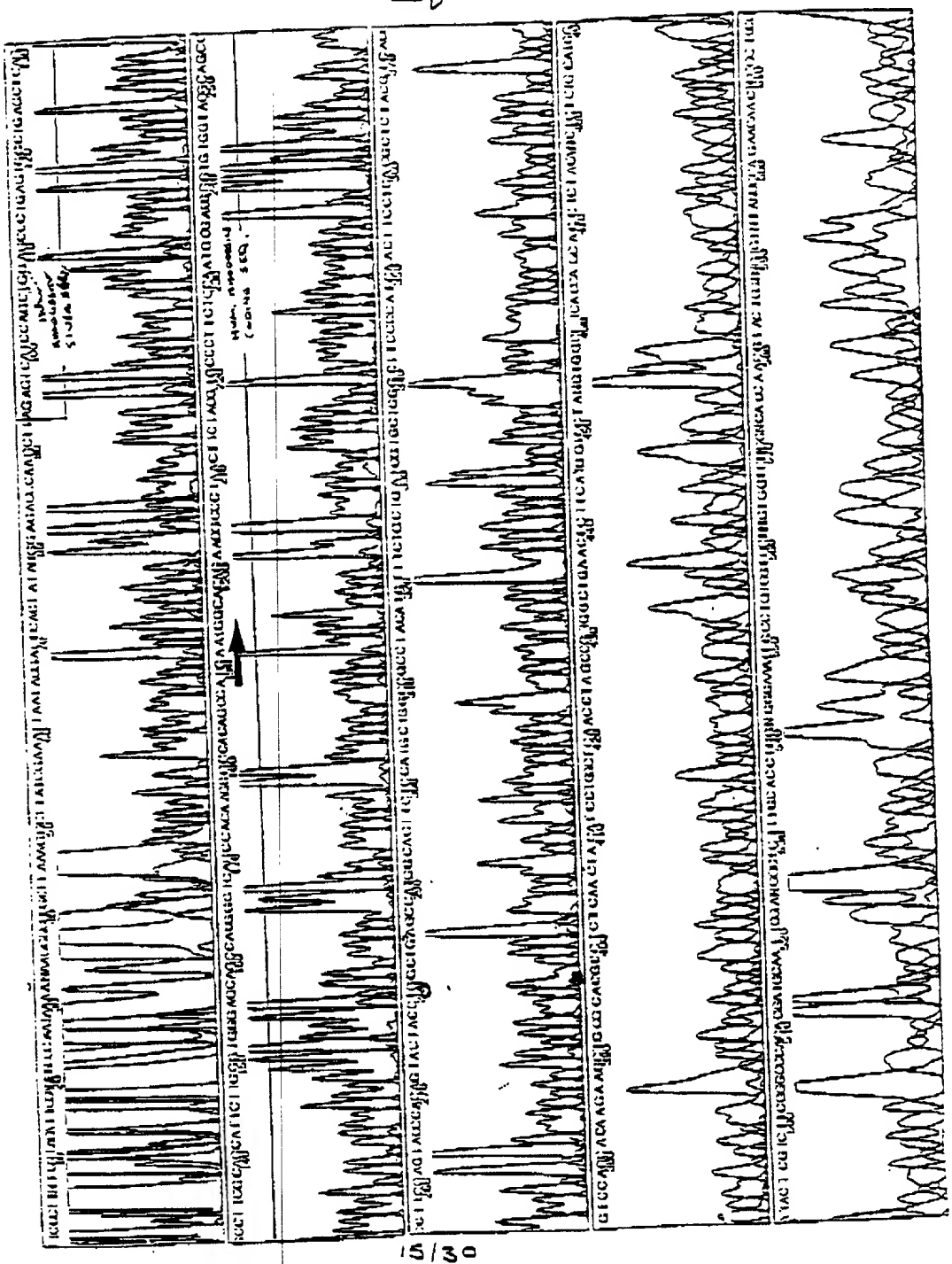


Figure 8 A + B





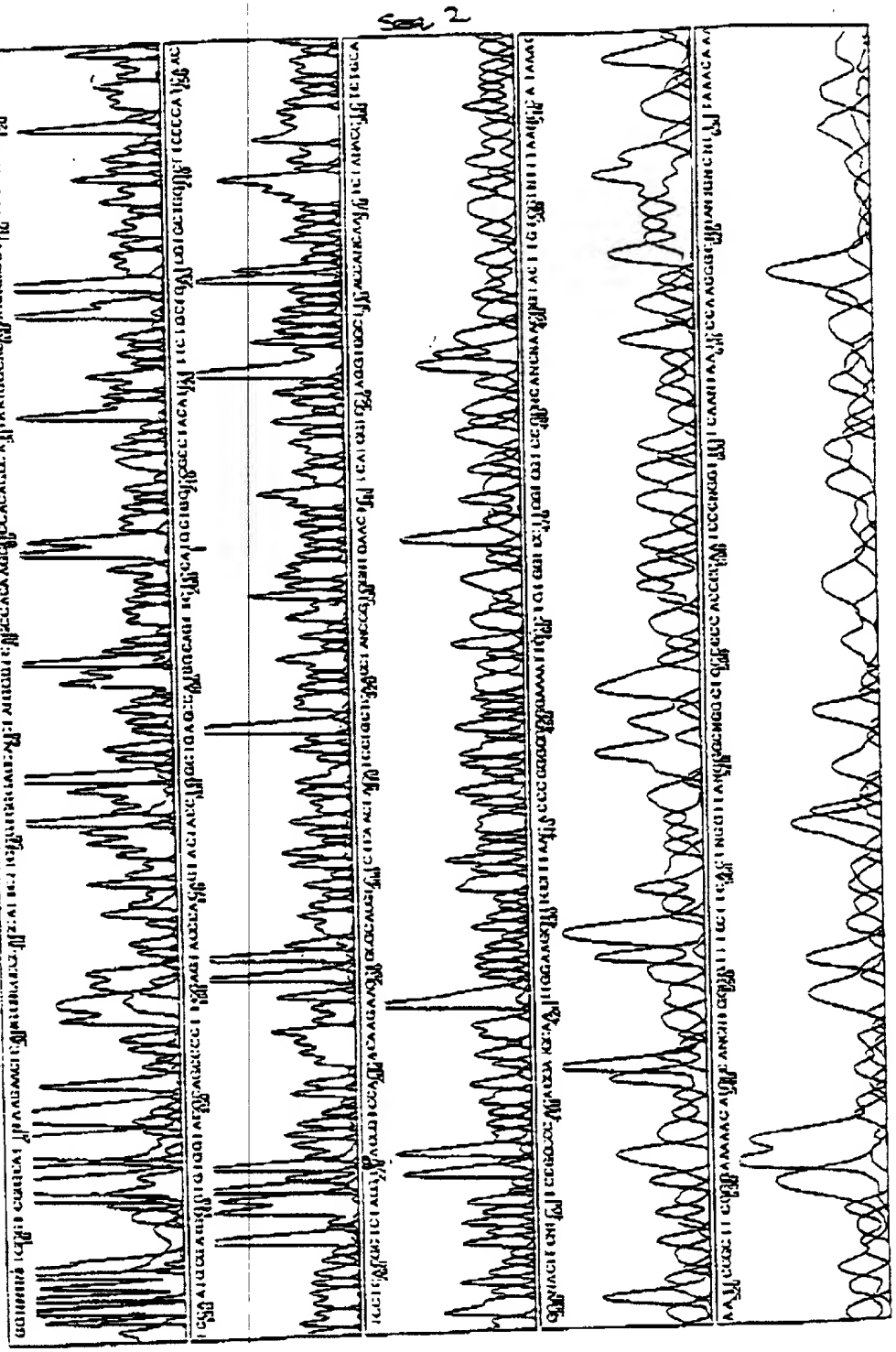
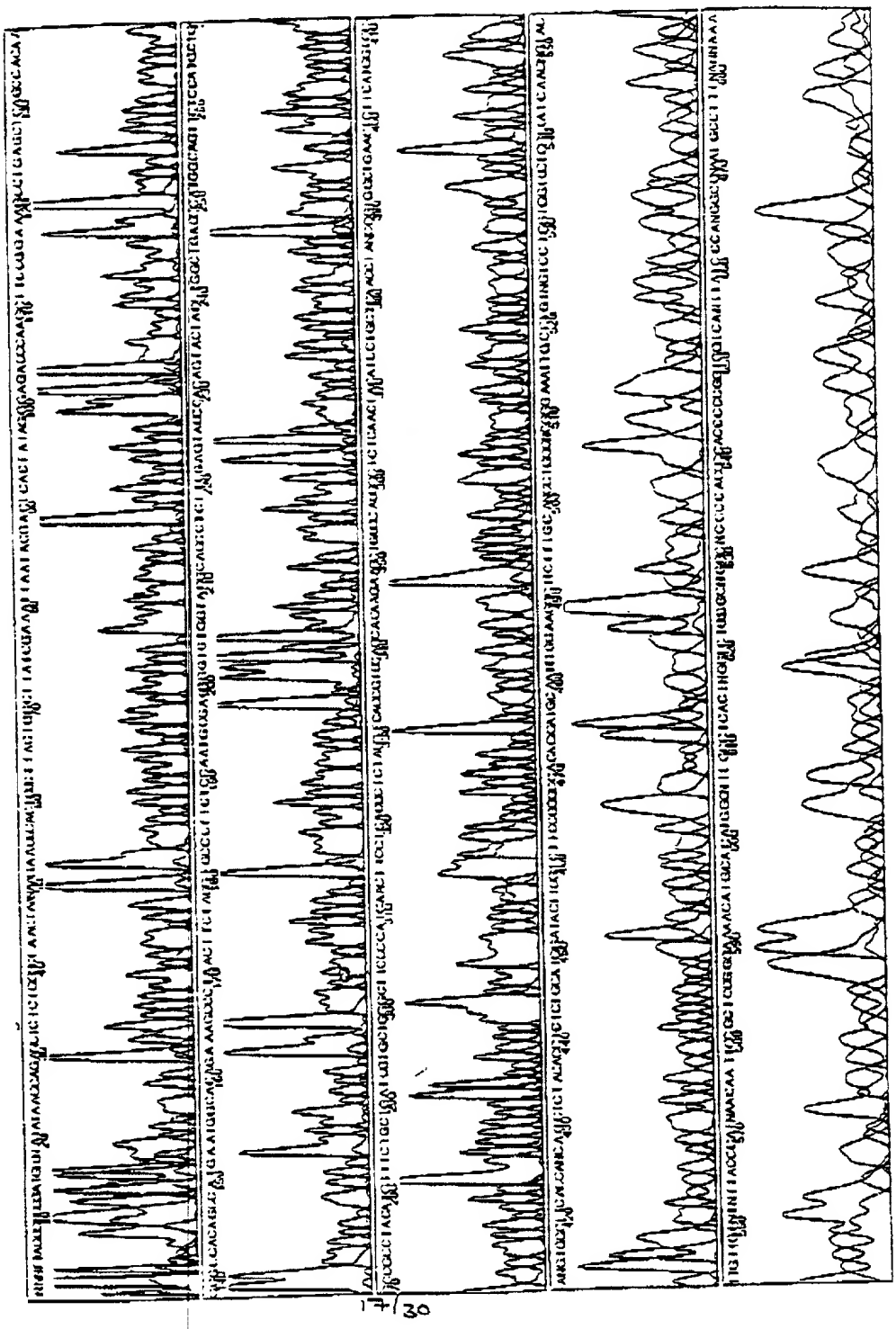


Fig. 3



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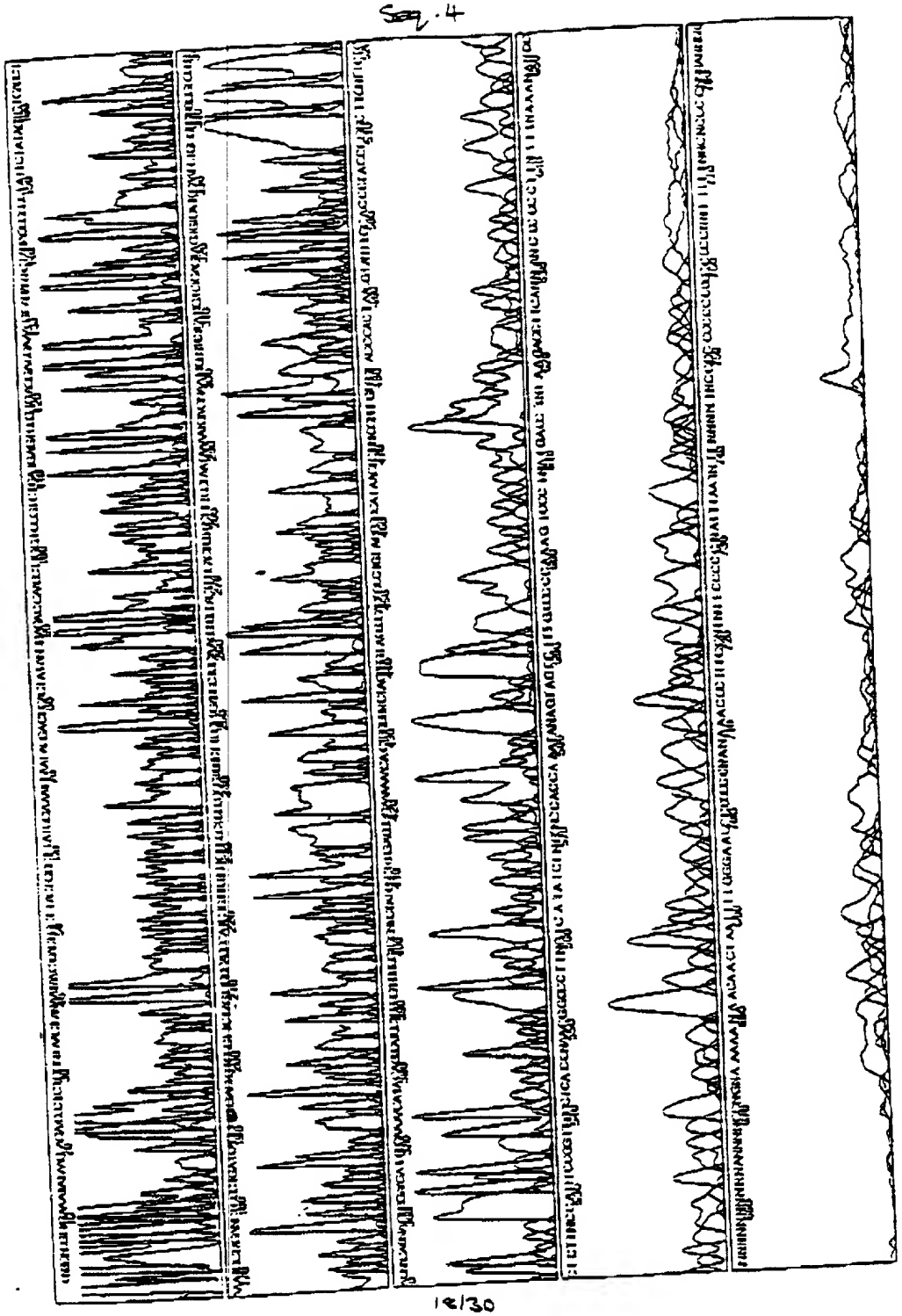

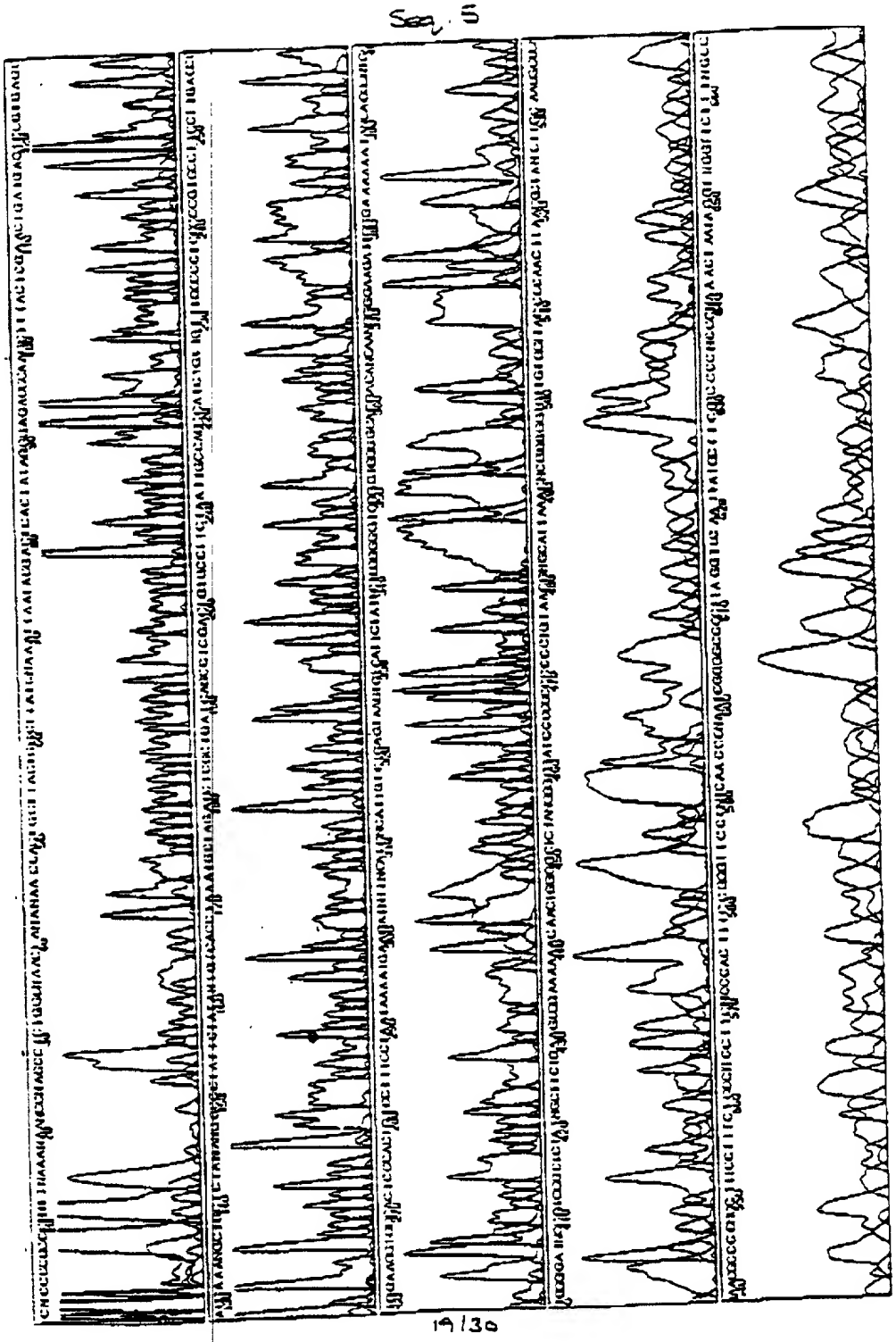

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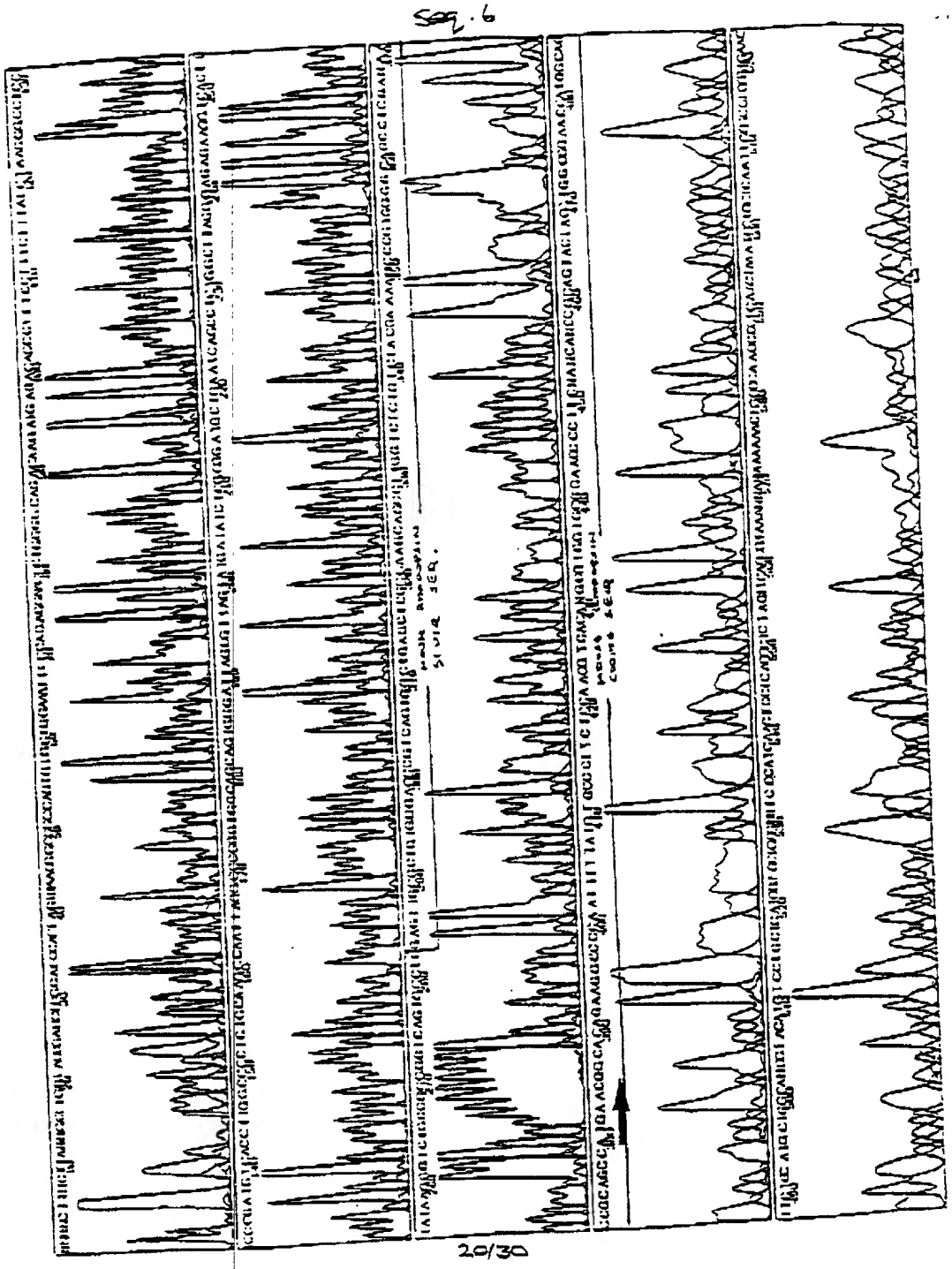
Fig. 6



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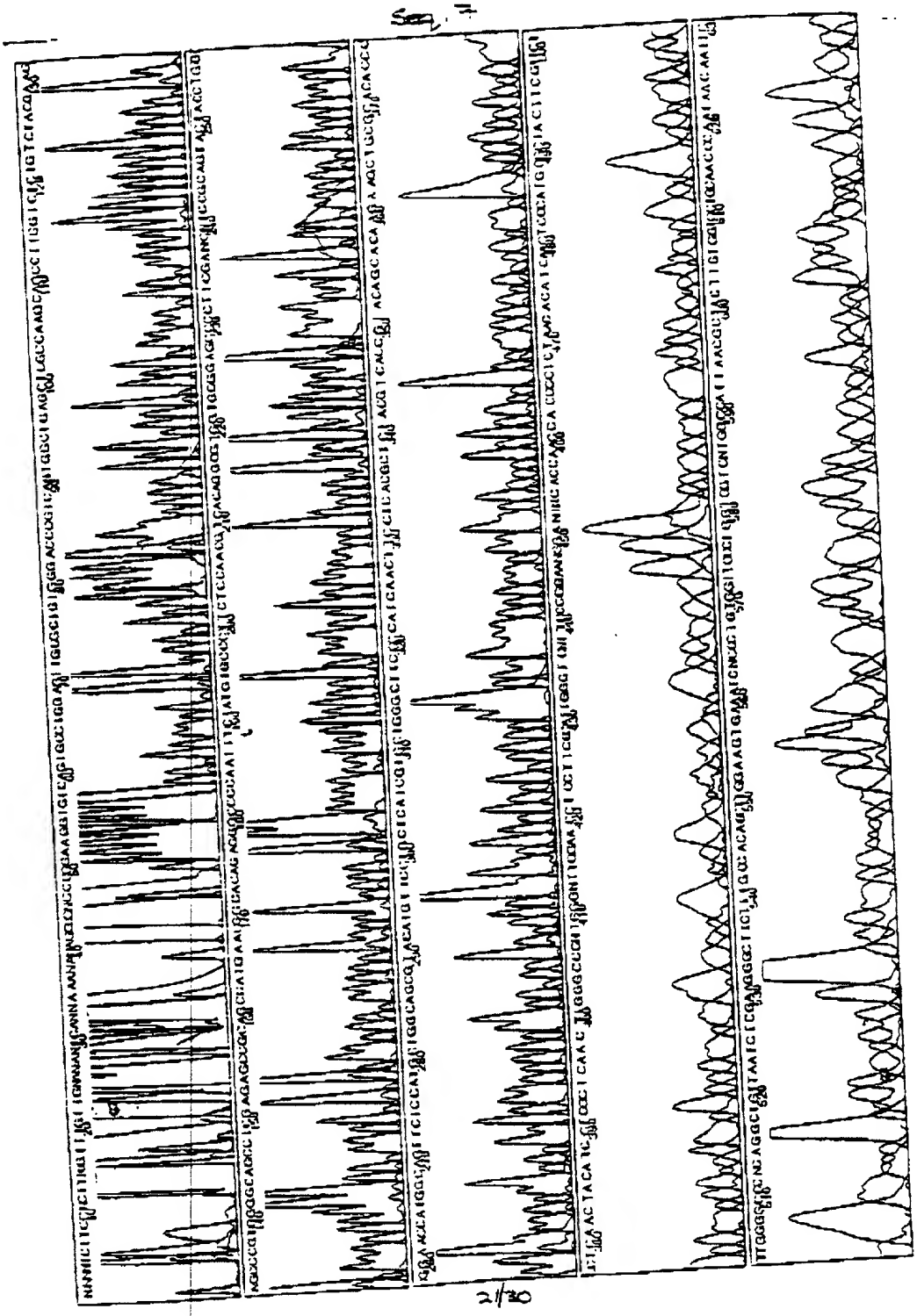
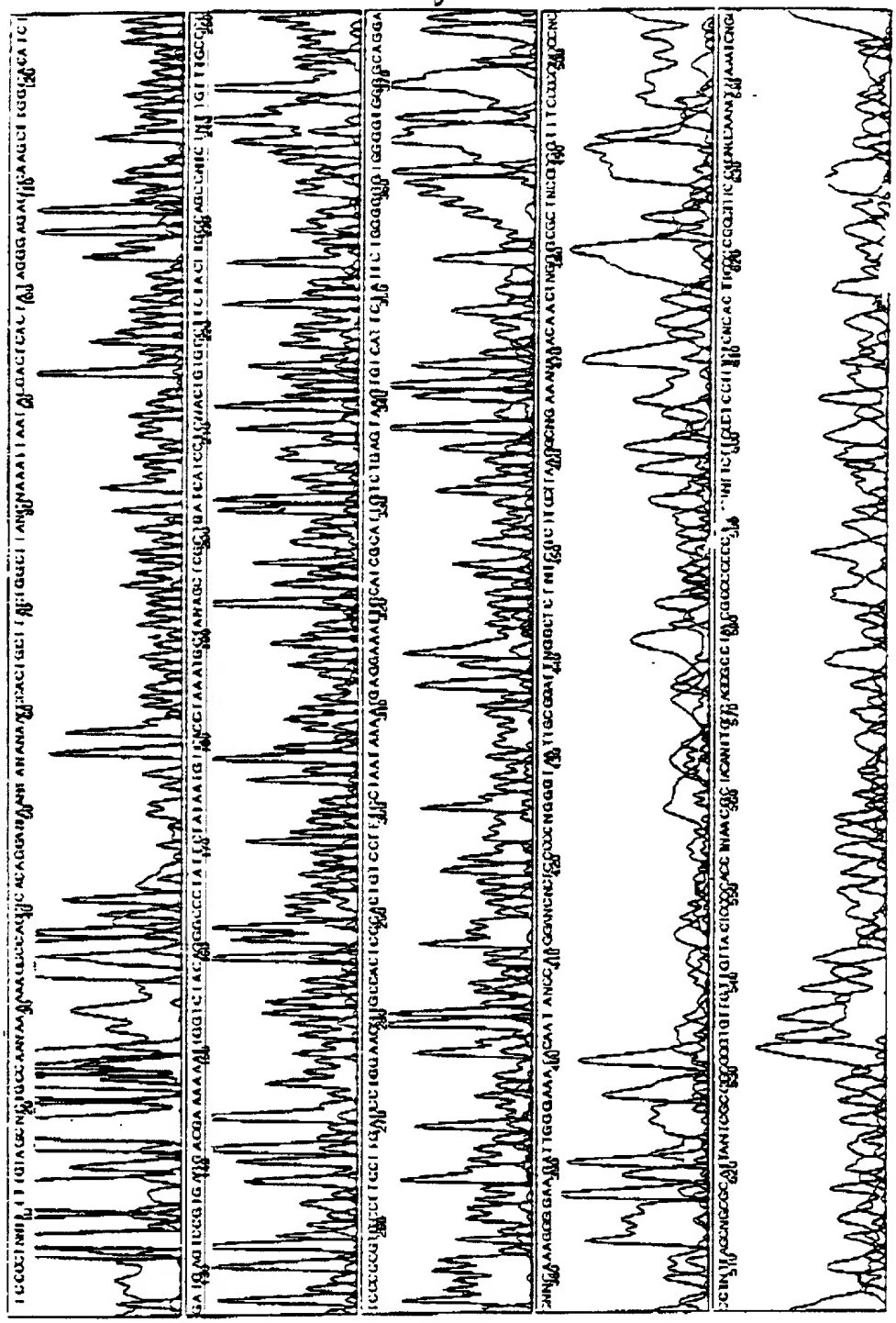


Fig. 8



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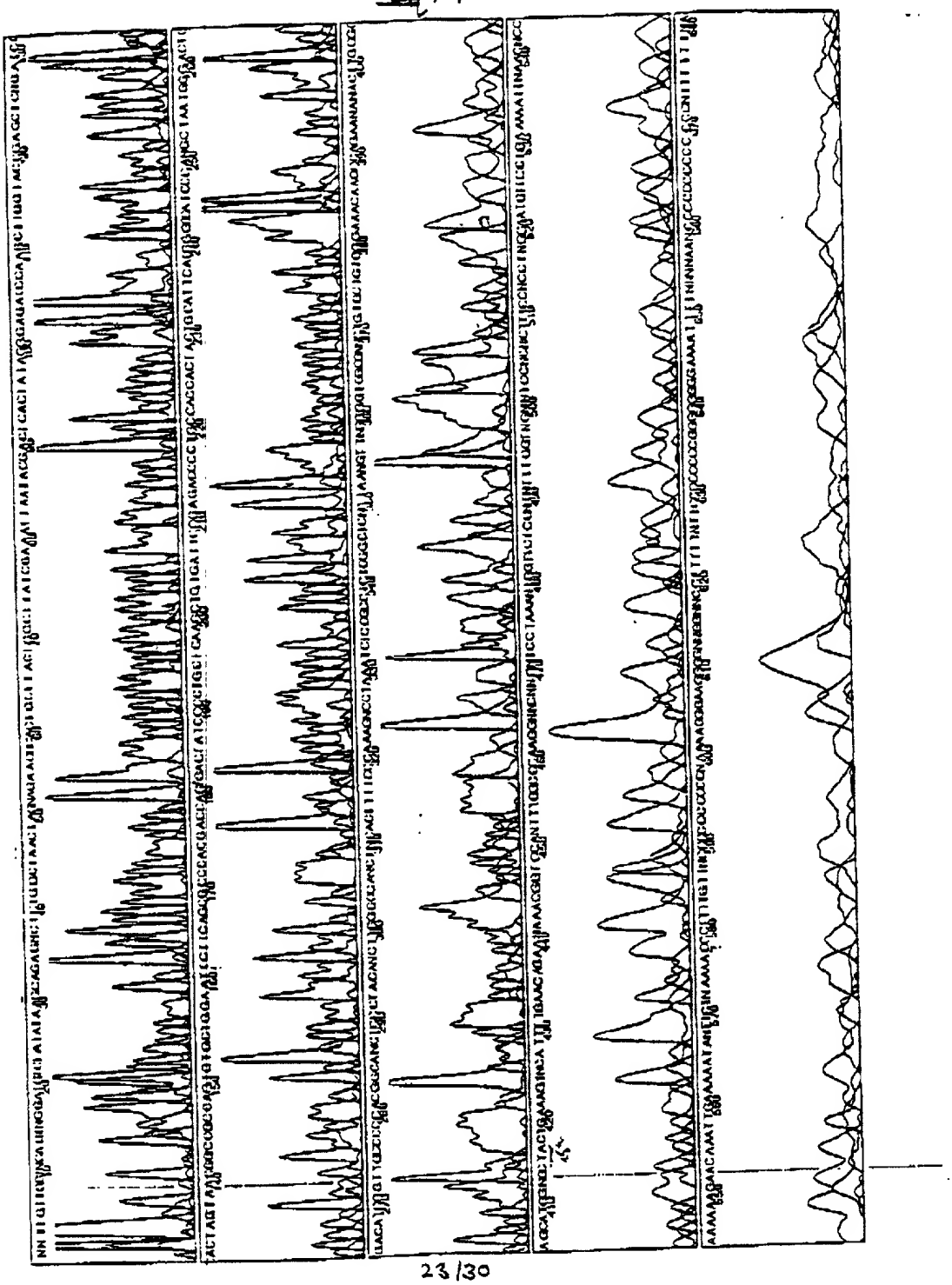
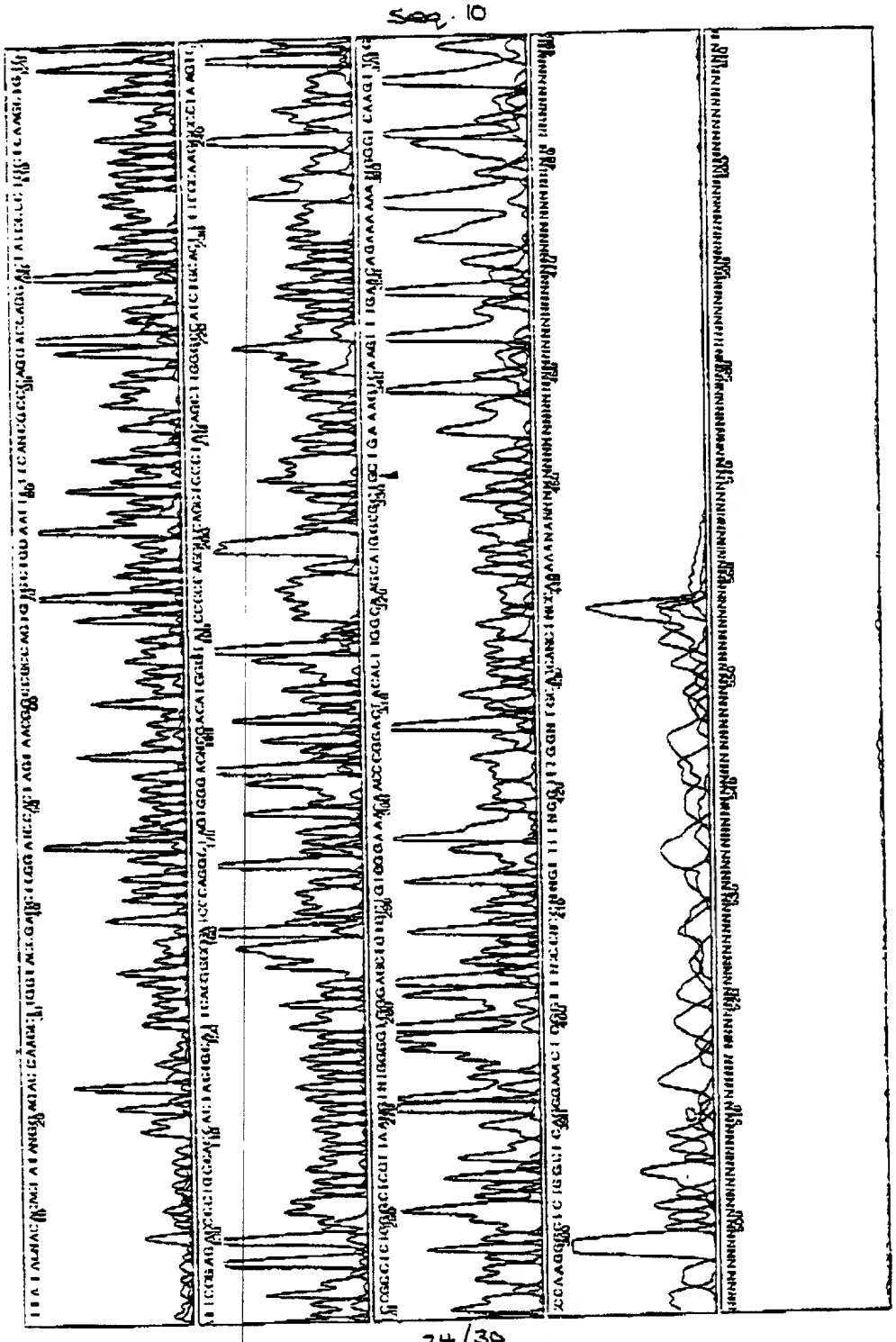


Fig. 10



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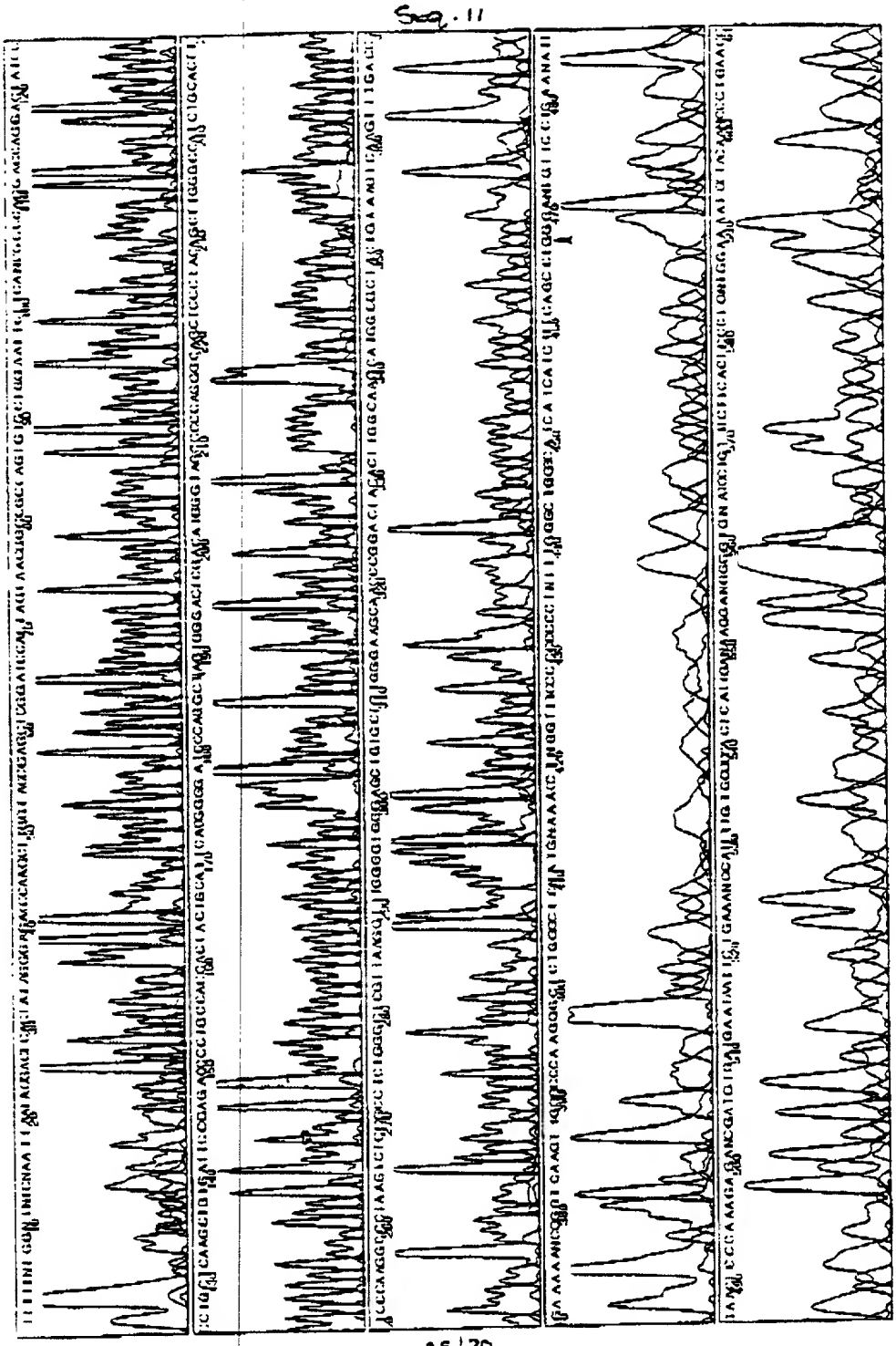


Fig. 12

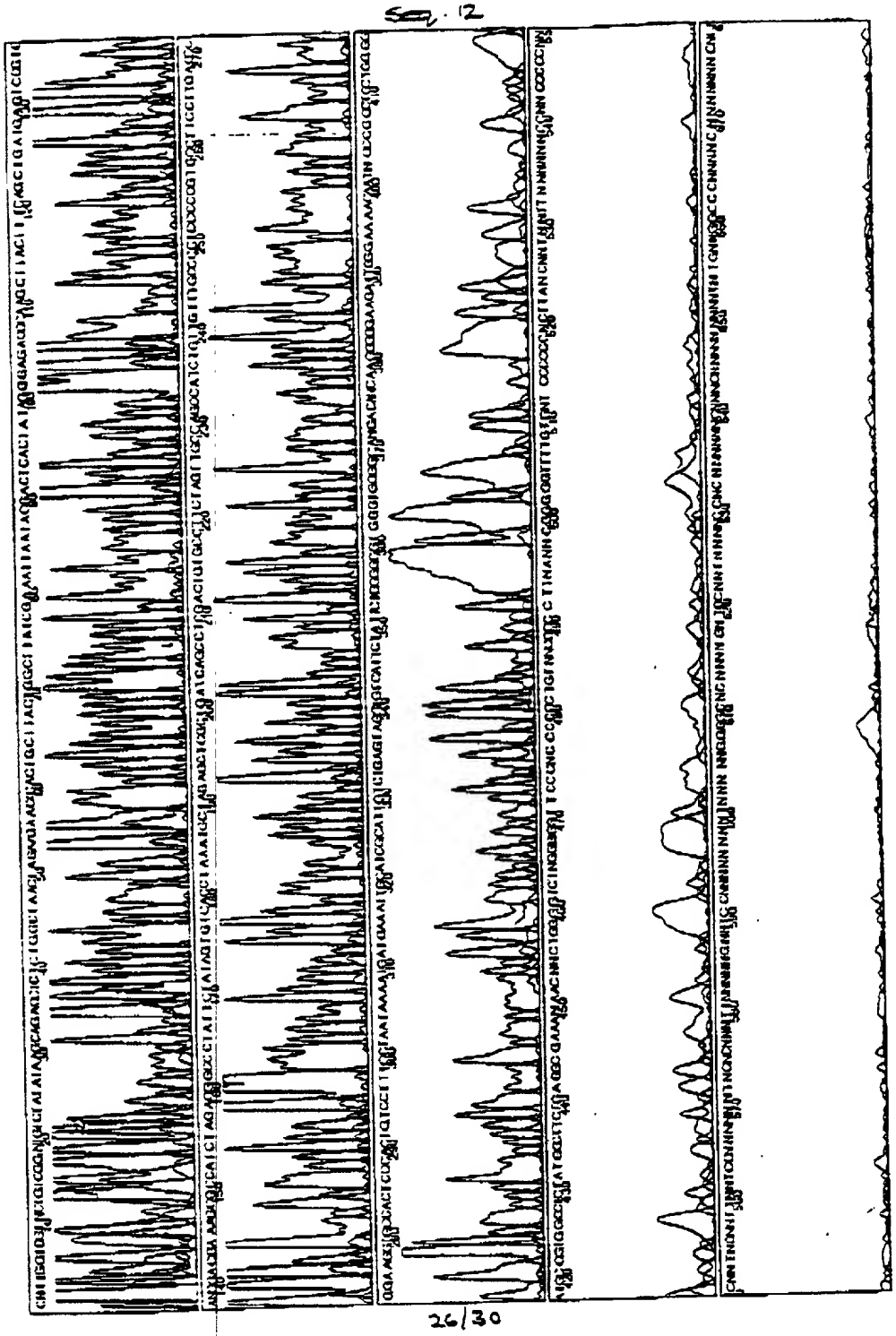


Fig. 13

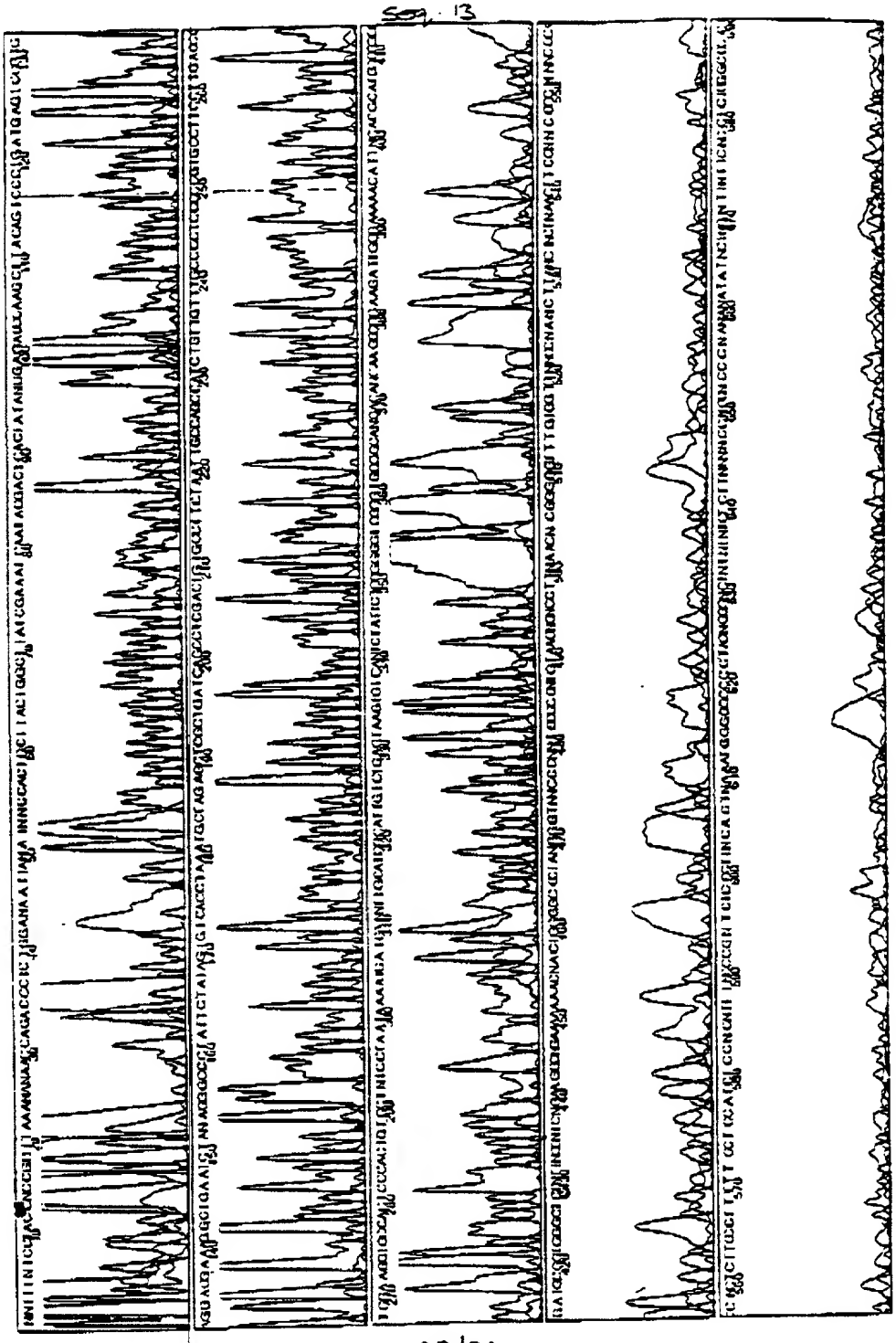
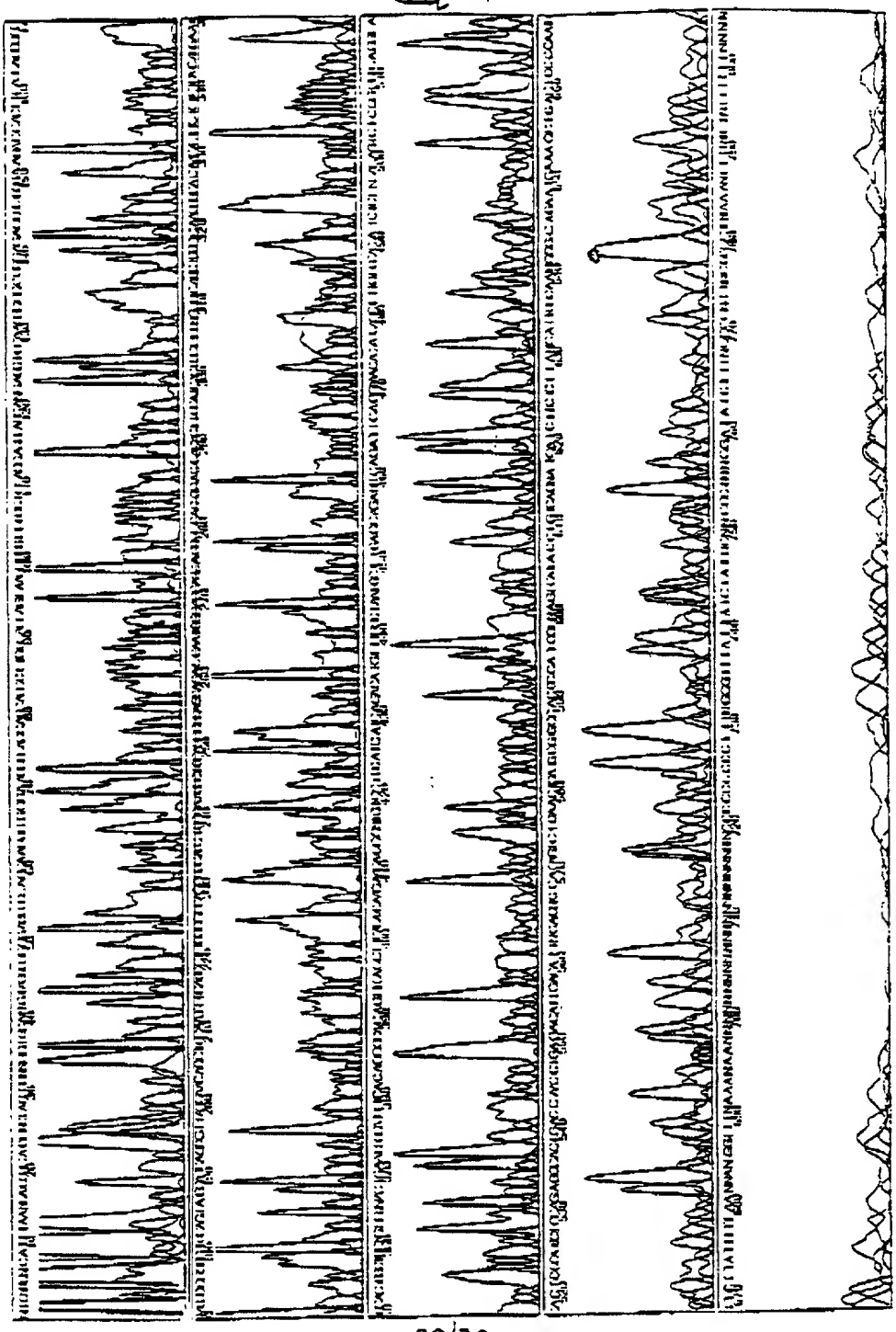


Fig. 14



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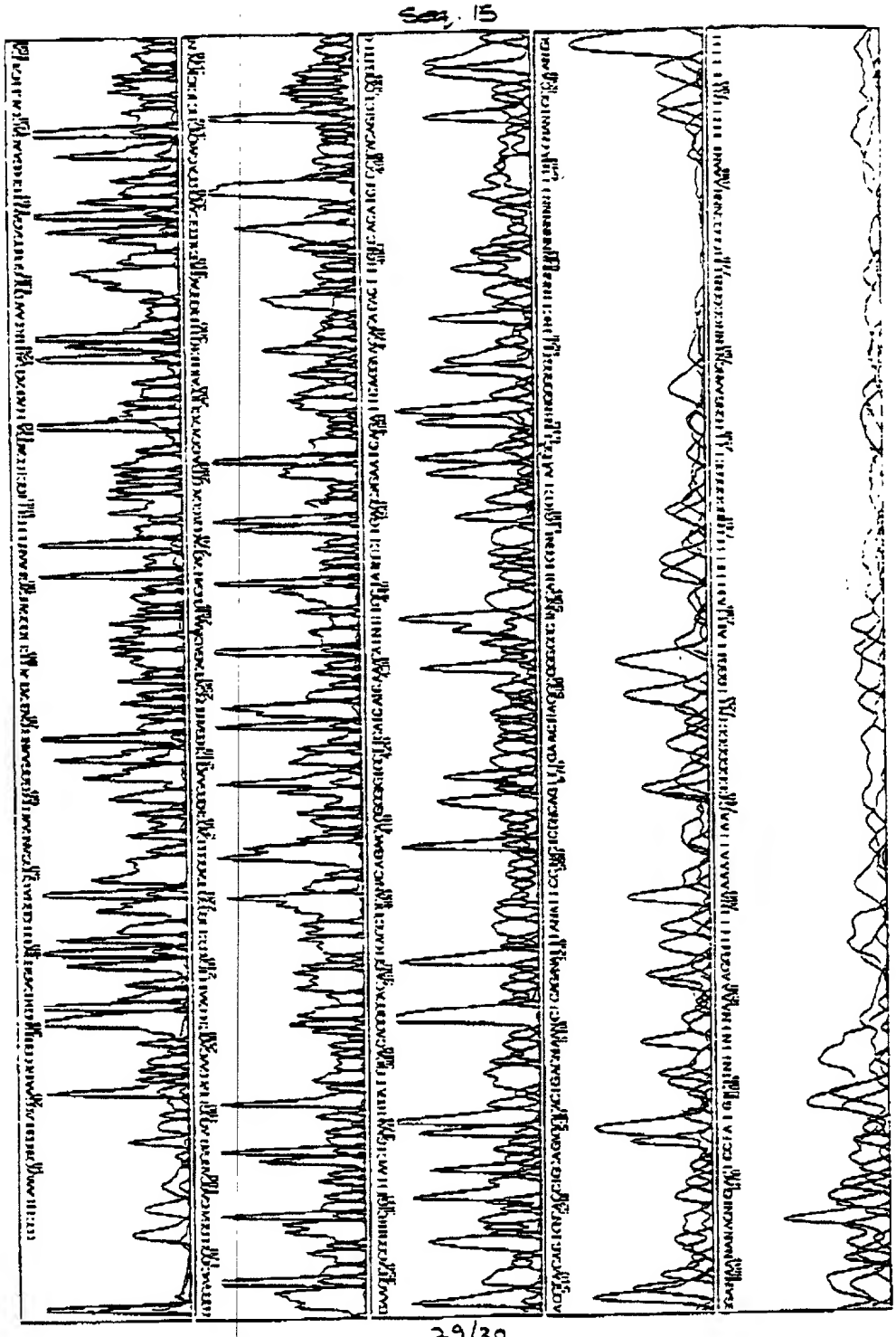

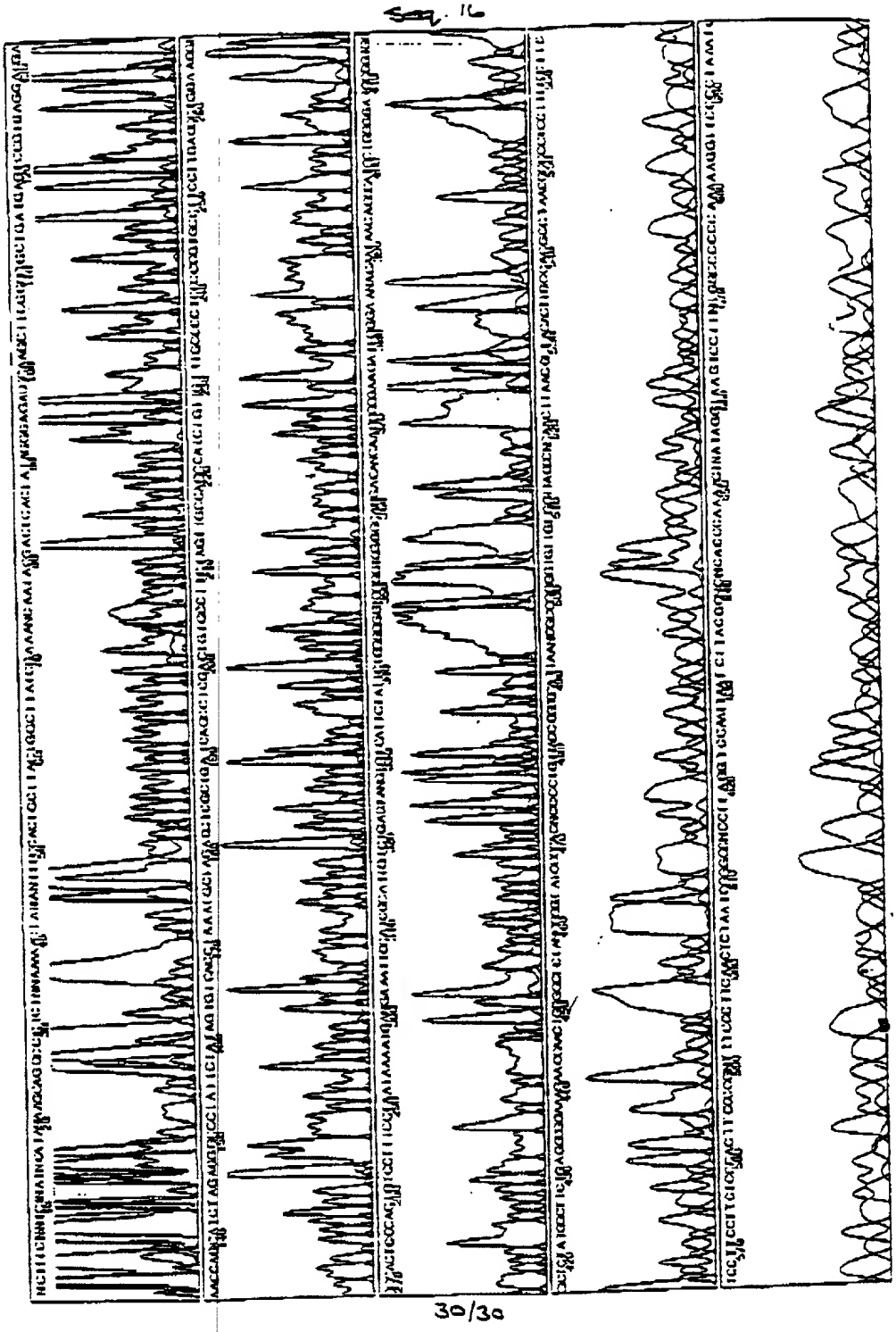

Declaration and Power of Attorney For Patent Application
English Language Declaration

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name,

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled GENETIC SUPPRESSION AND REPLACEMENT, the specification of which is attached hereto unless the following box is checked:

☒ was filed on 2 April 1997 as PCT International Application Number PCT/GB97/00929.

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to patentability as defined in 37 CFR § 1.56.

I hereby claim foreign priority benefits under 35 U.S.C. §119(a)-(d) or § 365(b) of any foreign application(s) for patent or inventor's certificate, or § 365(a) of any PCT International application which designated at least one country other than the United States, listed below and have also identified below by checking the box, any foreign application for patent or inventor's certificate, or PCT International application having a filing date before that of the application on which priority is claimed:

Prior Foreign Application(s)			Priority Not Claimed
9606961.2	GB	2 April 1996	☐
(Number)	(Country)	(Day/Month/Year Filed)	
____	____	____	☐
(Number)	(Country)	(Day/Month/Year Filed)	

I hereby claim the benefit under 35 U.S.C. § 119(e) of any United States provisional application(s) listed below.

____	____
(Application Number)	(Filing Date)
____	____
(Application Number)	(Filing Date)

I hereby claim the benefit under 35 U.S.C. § 120 of any United States application(s), or 365(c) of any PCT International application designating the United States, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT International application in the manner provided by the first paragraph of 35 U.S.C. § 112, I acknowledge the duty to disclose information which is material to patentability as defined in 37 CFR § 1.58 which became available between the filing date of the prior application and the national or PCT international filing date of this application:

MUR-7520

(Application Number)	(Filing Date)	(Status - patented, pending, abandoned)
(Application Number)	(Filing Date)	(Status - patented, pending, abandoned)

POWER OF ATTORNEY: As a named inventor, I hereby appoint the following attorney(s) and/or agent(s) to prosecute this application and transact all business in the Patent and Trademark Office connected therewith:

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Allan Ratner	Reg.No. 19,717	Robert L. Andersen	Reg.No. 25,771	Joshua L. Cohen	Reg.No. 38,040
Andrew L. Ney	Reg.No. 20,300	Christopher R. Lewis	Reg.No. 36,201	Christopher J. Dervishian	Reg.No. 42,480
Kenneth N. Nigon	Reg.No. 31,549	Louis W. Beardell, Jr.	Reg.No. 40,508	Jack J. Jankovitz	Reg.No. 42,680
Kevin R. Casey	Reg.No. 32,117	Rocco L. Adornato	Reg.No. 40,480		
Benjamin E. Lease	Reg.No. 33,412	Jacques L. Etkowicz	Reg.No. 41,738		
James C. Simmons	Reg.No. 24,842	Eric A. Dichter	Reg.No. 41,708		

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Address all telephone calls to: Allan Ratner at (610) 407-0700.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

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☒ Additional inventors are being named on separately numbered sheets attached hereto.

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Third inventor's signature Paul F Kenna Date 20/3/99

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IEX

Full name of fourth joint inventor, if any (given name, family name) ____

Fourth inventor's signature _____ Date _____

Residence ____

Citizenship ____

Post Office Address ____

Full name of fifth joint inventor, if any (given name, family name) ____

Fifth inventor's signature _____ Date _____

Residence ____

Citizenship ____

Post Office Address ____

Full name of sixth joint inventor, if any (given name, family name) ____

Sixth inventor's signature _____ Date _____

Residence ____

Citizenship ____

Post Office Address ____

Full name of seventh joint inventor, if any (given name, family name) ____

Seventh inventor's signature _____ Date _____

Residence ____

Citizenship ____

Post Office Address ____